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Dedication

This dissertation is dedicated to my nephews Jamil and Quinn Jackson. I want you both to know that this body of work is proof that dreams can become reality. I will always be in your respective corners. Remember to shoot for the moon, because if you miss, you will still land among stars.

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The greatest lesson I have learned in life so far is that it takes an entire village to raise a child. My own initiative and personal aspirations aside, there are so many people who have helped me in my accomplishments.

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Indianapolis was not where I envisioned my studies would take me. I have made so many connections that I know will carry on long after graduation. I have been blessed with too many to name, and I thank each of you for keeping me sane through the difficulties of graduate and medical school alike.

Yohance Mandela Allette
MODULATORY ACTIONS OF HMGB1 ON TLR4 AND RAGE IN THE PRIMARY AFFERENT
SENSORY NEURON

Damage Associated Molecular Patterns (DAMPs) act largely as endogenous ligands to initiate and maintain the signaling of both inflammatory processes and the immune response. Prolonged action of these endogenous signals are thought to play a significant role in sterile inflammation which may be, integral to the development of chronic inflammation pathology.

HMGB1 (High Mobility Group Box 1) is a highly conserved non-acetylated protein which is among the most important chromatin proteins and serves to organize DNA and regulate transcription. Following stress or injury to the cell, hyperacetylation of lysine residues causes translocation of HMGB1 and eventual release into the extracellular environment where it can take the form of a DAMP and interact with cell types bearing either the Receptor for Advanced Glycation End-products (RAGE) or Toll-Like Receptor 4 (TLR4). Activation of these surface receptors contribute directly to both acute and chronic inflammation.

This project investigated the role of HMGB1 through its receptors Receptor for Advanced Glycation End-products (RAGE) and Toll-Like Receptor 4 (TLR4) as it pertained to the development of chronic inflammation and pathology in small diameter, nociceptive sensory neurons. It was demonstrated that the neuronal signaling associated with exposure to HMGB1 is dependent upon the ligands conformational states, as the state dictates its affinity and types of neuronal response.

Neuronal activation by bacterial endotoxin or the disulfide state of HMGB1 is dependent on TLR4 and the associated signaling adapter protein, Myeloid differentiation primary response gene 88 (MYD88). Interruption of the receptor-mediated signaling cascade associated with MyD88 was shown to be sufficient to mitigate ligand-dependent neuronal activation and demonstrated significant behavioral findings. Further downstream signaling of HMGB1 in the neuron has yet to be identified, however important steps have been taken to elucidate the role of chronic neuroinflammation with hopes of eventual translational adaptation for clinical therapeutic modalities.

Fletcher A. White, Ph.D., Chair

Table of Contents

Chapter 1: Introduction	1
What is Pain.....	1
The Link between Chronic Inflammation and the Persistent Nociceptive Condition.....	3
Actions of Exogenous and Endogenous Ligands	8
High Mobility Group Box 1	10
The HMGB1 Receptors	20
TLR4	20
RAGE	26
The TIR Domain	33
Thesis Aims.....	34
Chapter 2: HMGB1 and Nociceptive Neuronal Signaling.....	37
Abstract	38
Introduction.....	39
Methods	42
Results	51
Discussion.....	57
Figure Legend	61
Chapter 3: Neuronal Excitation and the TIR Adaptor Proteins.....	70
Introduction.....	71
Methods	74
Results	79
Discussion.....	83
Conclusion	87
Figure Legend	88
Chapter 4: Project Discussion	94
The effects of HMGB1 on neuronal signaling	96
Summary and Conclusions	98
Chapter 5: Future Directions and Studies.....	103
The relationship between sodium channels and innate immune signaling	103
RAGE in traumatic brain injury.....	104
The role of gender in the study of pain and TLR4	106
HMGB1 and RAGE in cellular repair, proliferation and cancer	108
Appendix	110
References	111
Curriculum Vitae	

Chapter 1: Introduction

What is Pain

Pain is universal. It is a result of evolutionary development for a feedback system within an organism to signal harm. It exists to inform of a disturbance in homeostasis, alerting the organism to a circumstance that is not conducive to survival (Woolf and Salter, 2000). The word 'pain' originates from Latin for punishment, often from a supernatural origin and an indictment of the individual, and their suffering is a trial that must not be interfered with by others (Morris, 1998). This experience is usually transitory (acute pain) lasting only until the noxious stimulation is removed or healed. Some painful conditions may persist for years (chronic pain). Though both of these conditions may seemingly exist in the world of multicellular organisms, the term pain is typically associated with a subjective experience which is not present in the non-humanoid world. In its place is the term nociception. Nociception was coined to distinguish the physiological process from the subjective experience (pain), and can generally be categorized into two separate entities, acute and persistent (Sherrington, 1973 #3699). The sensation associated with superficial injury, or acute nociception, indicates a clear need for attention, medical or otherwise. Persistent nociception, is typically much more severe in nature, and is exemplified by inflammatory or pathological events.

Both pain and nociception exist as critical alarm systems to alert organisms to threats and are essential for initiation of escape behaviors (Woolf and Salter, 2000). The functional process of recognizing harmful stimulation (or nociception) is transduced by a nociceptor which can detect thermal, mechanical, and chemical sensations. The majority

of nociceptors are classified by which of the environmental modalities they respond to such as heat and pressure (Dubin, 2010 #3708). However, some nociceptors respond to more than one of these modalities and are consequently designated polymodal. Other nociceptors respond to none of these modalities (although they may respond to stimulation under conditions of inflammation) are referred to as sleeping or silent.

Traditionally, the distinction between acute and chronic nociceptive conditions in animals and persistent pain in humans has relied upon an arbitrary interval of time from onset. The two most commonly used markers that distinguish acute and chronic pain are 3 months and 6 months since the onset respectively. Some theorists and researchers have placed the transition from acute to chronic nociceptive conditions at 12 months (Katz and Seltzer, 2009, Voscopoulos and Lema, 2010). For the purpose of attributing observations of rodent models of peripheral nerve injury-induced nociceptive conditions, the chronic condition of nociception can be defined by when duration extends beyond the expected period of healing. The condition of chronic pain in humans can be viewed as a subjective experience that outlives its original causes which can worsen over time and become a disease in its own right.

As a disease, the human persistent pain condition is one of the most wide-reaching and debilitating afflictions of the patient population. In its various incarnations, persistent pain is typically disregarded as merely a symptom of a greater issue. Medically, it is simply another clue in clinical detective work of the 'true' underlying pathology that needs to be treated. From a cultural standpoint, the chronic pain condition is often something to be dismissed when expressed, and not treated as a viable malady (Eccleston, 2001). One

study revealed that in a population suffering from chronic pain, over a third expressed personal issues of self-esteem, and internalized stigma due to use of medications for pain relief (Asmundson et al., 1996, Eccleston, 2001). Another study describes that pain-related fear can actually prove more damaging and weigh heavier on the patient than experiencing the chronic pain they originally feared (Crombez et al., 1999). These psychological findings mirrored changes in cognitive function with these same patients demonstrating lower feelings of control over their individual situation, which permeates into other aspects of life (Eccleston, 2001).

The human chronic pain condition has been found to affect over 100 million patients in the U.S. health care system, with medical costs and expenditures greater than \$550 billion dollars annually (Dzau and Pizzo, 2014). These numbers fall significantly short of global totals, emphasizing that pain in all its manifestations, and its treatment methods, equate to a worldwide healthcare issue. Furthermore, as medicine and bio-medical research continue to advance and extend life expectancy, they also increase the presence of pain and other chronic maladies within the patient population (Oeppen and Vaupel, 2002, Vaupel and KG, 2005).

The Link between Chronic Inflammation and the Persistent Nociceptive Condition

Inflammation is the mechanism by which an organism responds to injurious stimuli, exogenous or endogenous in nature, in an effort to maintain homeostasis. This mechanism is a complex compilation of temporal and mechanical structures and events, requiring the proper function of multiple cell types and precise intercellular

communication via multiple ligands and both pro and anti-inflammatory mediators (Kumar et al., 2005, Medzhitov, 2010). The resolution of inflammation may be the most important aspect of the process. Although advantageous and productive for the survival of an organism, unmitigated inflammatory signaling or unresolved processes of inflammation can have severe health consequences leading to the development of multiple diseases types (Nathan and Ding, 2010). Chronic inflammation can result in the development of arthritis, sepsis/septic shock, asthma, multiple sclerosis, atherosclerosis, and pain (McFarland and Martin, 2007, Waldburger and Firestein, 2009, Van-Assche et al., 2011, Chung, 2012, Feldman et al., 2012, Sankowski et al., 2015). In fact, one of Virchow's cardinal signs of inflammation is pain. When unresolved, the beneficial and necessary process of inflammation transforms into the maladaptive cascade of chronic inflammation.

The acute inflammatory response is largely sustained by the presence of constant stimulation, be it based on a pathogen or disease entity. The resolution of inflammation is typically an active process. It requires a number of mechanisms, including the production and release of anti-inflammatory cytokines, down-regulation of pro-inflammatory cytokines, and the desensitization of the associated receptors in the affected tissue or organ system (Chandrasoma, 1997, Medzhitov and Janeway, 1998). In the absence of these regulatory inflammatory processes, a state of chronic inflammation can result. The transition from acute to chronic inflammation is not well understood, especially given that the regulatory and resolution mechanisms of acute inflammation are not fully defined.

Chronic inflammation is abnormal and does not benefit the body. The disease process of chronic inflammation can be described as simultaneous circuits of destruction and healing, both preventing the other from coming to a halt (Kumar et al., 2005). As the process carries on, cytokines, chemokines, and other related signaling ligands are continued to be released and evoke response via multiple receptors and cells, resulting in a feed-forward mechanism (Heap and van Heel, 2009). Chronic inflammation has been connected to multiple immune disorders and non-immune system disease outside of the aforementioned chronic pain. This includes, but is not limited to, cancer (in its many variations), heart disease, traumatic brain injury, several autoimmune disorders, and diabetes (Kang et al., 2010, Mohammad et al., 2012).

What the two processes of acute and chronic inflammation have in common is the need for a starting point. Often in error, pathogenic or exogenous sources are assumed for the initiation of an inflammatory response. Although inflammation can indeed arise via pathogenic means, this is not to exclude an internal, or endogenous, starting point for the inflammatory process (Chen and Nunez, 2010). In relation to the aforementioned topic of chronic pain and inflammation it would be logical to surmise that an endogenous ligand is responsible for the inflammatory cascade in the development of a chronic pain state.

Chronic nociceptive conditions and chronic inflammation share a loss of a functional purpose; existing only as maladaptive processes to the host organism. Interestingly, evidence continues to build in the literature supporting the theory that chronic nociceptive conditions are actually products of chronic inflammation. This is

further grounded in the observations that both the immune system and the nervous system developed and evolved in concert with one another, encouraging communication between the two systems (Sankowski et al., 2015). Once considered to be 'immune privileged' and not a benefactor or recipient of the inflammatory process, the nervous system has now been identified both exhibiting contributing to inflammation (Lucas et al., 2006).

In one study of experimental colitis in a murine model, the stimulation of cholinergic nerves (e.g. vagus nerve, splenic nerve) through agonists or administration of acetylcholinesterase inhibitors led to lower levels of inflammatory mediators and reduced mucosal inhibition, a marker of inflammatory severity (Ji, 2014 #2988; Borovikova, 2000 #3714). The ligation or removal of these key neuronal structures resulted in a diminished anti-inflammatory profile in the observed tissue (Ji et al., 2014a). This example falls under the neuronal-controlled cholinergic anti-inflammatory pathway. This pathway results in neurons releasing the signaling compound noradrenaline, which in turn activates splenic T-cells and continues the signal cascade to the effect of Tumor Necrosis Factor- α (TNF α) downregulation (Rosas-Ballina et al., 2011). Interestingly, the nervous system is particularly vulnerable to the process of inflammation, as seen on a systemic level in severe septic patients (Sankowski et al., 2015). These same patients demonstrate a susceptibility to nervous system dysfunction, with the development of cognitive impairment in over eighty percent of patients (Ely et al., 2004).

Many of the cytokine and chemokine signaling pathways of chronic inflammation have been observed to play a role in the mitigation of pain, originating from both neuronal

and non-neuronal sources, and have been shown evoke peripheral neuronal sensitization, and elicit persistent nociceptive responses in various circumstances (White et al., 2007, Okun et al., 2011). Currently, the literature fully endorses that products of the inflammation associated with tissue insult or injury can indeed directly affect nociception (Ji et al., 2014b, Schaible, 2014). The inflammatory mediator interleukin-1 β has been shown to directly signal through nociceptive neurons, and TNF α when introduced either locally or systemically can result in both thermal and mechanical hyperalgesia (Cunha et al., 1992, Binshtok et al., 2008). These are only a few of the examples of inflammatory products affecting nociceptors and eliciting a neuronal response.

The mammalian nervous system has shown to be reactive to numerous inflammatory mediators and signaling compounds. This responsivity on the part of the neuronal system allows for the identification of these inflammatory signals on scale larger than the cellular level, through the generation of pain, alerting the nervous system and innate immune cells to the presence of microbial aggressors, and possible modification of neuronal characteristics (Hensellek et al., 2007, Mina-Osorio et al., 2012, Chiu et al., 2013). Thus, in order to better understand and eventually treat chronic pain effectively, it would be beneficial to study its relationship with inflammation as well as use chronic inflammation as a model for understanding chronic pain.

Similar to nociception, inflammation exists in two forms, acute and chronic. Generally, inflammation can be described as an organism's response to offensive stimuli or insult. However, this is more descriptive of the acute inflammatory process exemplified by innate immunity.

Actions of Exogenous and Endogenous Ligands

Stimuli for inflammation can be classified into two separate groups, pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). Both PAMPs and DAMPs can initiate and perpetuate the host immune response, with the main difference being the signal's origin. PAMPs are indicative of invading microorganisms and initiate the host's innate immune response to protect itself from infection (Janeway and Medzhitov, 2002). PAMPs are recognized by cells in the immune system via pattern recognition receptors, or PRRs, a class which includes the Toll-like receptor (TLR) family that upon activation, can initiate several inflammatory signal pathways, an example of which being nuclear factor- κ B (NF- κ B) (Bianchi, 2007). These receptors are particularly attuned to detection of foreign elements, or conserved 'non-self' ligands, usually of microbial origin.

DAMPs, on the other hand, are endogenously produced signals and are often referred to as 'alarmins'. As their alternate name suggests, they serve as an internal alarm system, and bring attention to cellular damage and stress in the absence of exogenous injury or infection. DAMPs range greatly in structure and function, due to their roles in biological homeostasis outside of inflammation (Tang et al., 2012b). The majority of these molecules activate the immune system's inflammatory response upon their extracellular release. This extracellular release only occurs in the face of cellular necrosis, or stress arising from injury or insult to the tissue; apoptosis will not result in the release of functioning alarmins into the extracellular compartment (Bianchi, 2007). The regulated and controlled clearance of apoptotic cells results in a subsequent halt in cellular activity

and inflammatory processes, making apoptosis the ‘most desirable’ form of death courted by inflammatory resolution (Savill, 1997, Rossi et al., 2007). Apoptosis results in chromatin condensation, a decrease in cytoplasmic volume, and other processes focused on the containment of DAMPs and to prohibit unwarranted inflammatory signal cascades (Kroemer et al., 2009). Cell death via necrosis, pyroptosis (caspase-1 dependent), and NET-osis (disintegration of the nuclear envelope), have been observed to activate inflammation through the release of nuclear materials and other pro-inflammatory mediators (Alessandri et al., 2013).

The subsequent inflammatory response as a result of DAMP-mediated activity is known as ‘sterile inflammation’, given the absence of any pathogen or exogenous microbial signal. Sterile inflammation serves as a mechanism of protection from UV radiation, noxious heat exposure, and physical trauma (Paterson et al., 2003, Candeias and Testard, 2015). Categorically, DAMPs include Heat Shock Protein (HSP) 70 and 90, S-100 proteins, oxidative-modified lipids, and members of the High Mobility Group protein family (Bianchi, 2007, Jin and Lee, 2008, Rohde et al., 2010).

As previously stated, the chronic inflammatory response closely mimics the maladaptive process of chronic pain, and vice versa. Thus, the concept of chronic sterile inflammation in particular makes for a more focused and interesting target of study to better investigate chronic pain. Sterile inflammation provides a process for the inflammatory products linked to chronic pain to be produced *en masse* and, given a problem in inflammatory regulation, also provides the possible positive feedback loop for chronic pain to progress and develop within the organism. However, with a possible

process defined, a culpatory ligand still needs to be identified for the continued nociceptive activity of chronic pain.

High Mobility Group Box 1

Survival for any organism is predicated upon eliciting the proper response. This mantra extends to the intracellular compartment, and involves a cell's ability to both send and receive signals from one another. One signal of particular interest is the chromatin protein High Mobility Group Box 1, or HMGB1. In the wake of cellular stress, injury, or death, HMGB1 can be released into the extracellular milieu triggering the rapid action of the innate immune system. This nature succinctly defines the role of many DAMPs, with its functionality within an organism fluidly changing according to the current cellular environment.

Formerly known as the molecule Amphoterin, HMGB1 has a molecular mass of approximately 27 kilodaltons, is devoid of any discernable enzymatic activity, and demonstrates the ability to moderate a wide variety of signaling pathways (Parkkinen et al., 1993, Bianchi and Manfredi, 2007). The molecule is highly conserved across all species, and is found in nearly all nucleated cell types (Klune et al., 2008). The first known role of HMGB1 was in neurite outgrowth, with its original nomenclature of amphoterin derived from its noted dipolar nature (Merenmies et al., 1991). Knockout of the HMGB1 gene has been shown to be a lethal mutation, signifying that the nascent role of the molecular ligand is integral to cellular homeostasis outside of its inflammatory capabilities (Calogero et al., 1999). HMGB1 is a member of the HMG family of nuclear proteins, a family that

possesses greater than 80% sequence identity conservation, and was originally identified with three distinct subsets (Bustin, 2001, Yang et al., 2013). These subsets are based upon an individual proteins' sub-domain, the box domain for HMGB proteins, the 'AT-hook' for HMGA, and the nucleosomal binding domain for HMGB-N (Bustin, 2001).

The HMG-box of HMGB1 acts as a DNA/RNA binding domain under naïve conditions (Stros, 2010). The molecular structure of HMGB1 is composed of three distinct domains, a negatively charged carboxyl terminus (C-terminus) and the 'A' and 'B' domains. The 'A' and 'B' domains bind in a transient fashion to the minor groove of DNA, which leaves the C terminus free to interact with transcription factors (TF) such as HOX proteins or p53, TATA binding proteins, and the core histones (Joshi et al., 2012). The binding between HMGB1 and DNA is for facilitation of structure modification, repair, and replication of the DNA (Andersson et al., 2002). In a similar fashion to the histone protein H1, HMGB1 demonstrates preferential binding to what is known as alternative DNA structures. Alternative DNA structures, also known as non-B type DNA, includes bent, misshapen, unwound, or kinked DNA (Stros, 2010). This preference for alternative DNA structures most likely arises from a desire at the cellular level to minimize the possibility of genetic mutations that could endanger the organism as a whole (Jackson and Bartek, 2009). Unlike H1, HMGB1 binding to the DNA constructs results in the HMGB1 molecule severely bending the corrupted genetic material (Thomas, 2001, Lange and Vasquez, 2009).

The structural composition of HMGB1 is intriguing in its complexity. HMGB1 is lysine-rich, making it adept at binding negatively charged compounds such as the protein

heparin and DNA, as well as providing a key target for post-translational modifications. As the study of HMGB1 continues, what becomes increasingly clear is the high level flexibility of the molecule, not only in a structural capacity, but function when one considers the specificity introduced upon post-translational modification.

Further investigation of the 'A' and 'B' domains reveal their respective anti-inflammatory and pro-inflammatory functionality (Yang et al., 2004, Tsung et al., 2014). Interestingly, the 'A' domain in particular can act in an anti-inflammatory capacity in an antagonistic response to the pro-inflammatory signaling of HMGB1 or the truncated 'B' domain (Andersson et al., 2002, Yang et al., 2004).

Prior to being secreted from the nucleus of the cell, post-translational modifications of HMGB1 affects the molecule's ability to bind to DNA and affect histone function, in addition to crossing both the nuclear and cytoplasmic membranes. Within the nucleus, HMGB1 can be found in either a hyper or hypo-acetylated states. HMGB1 acetylation takes place at lysine residues 2, 11, and 81 (Sternier et al., 1979, Bonaldi et al., 2003, Assenberg et al., 2008, Elenkov et al., 2011). In particular, the lysines at positions 2 and 11 are hypothesized to be critical for HMGB1 'A' domain to bind to distorted DNA structures (Assenberg et al., 2008). Hypo-acetylation restricts HMGB1 to the nucleus to continue in its role of DNA repair and replication (Polanska et al., 2014). Hyper-acetylation of HMGB1 shifts its responsibility from a protein of nuclear maintenance to one of extracellular signaling (Yang et al., 2013). The mechanism by which hyper-acetylation occurs has been hypothesized to involve deacetylase inhibitors presented from fibroblasts to reduce the overall nuclear import of HMGB1. The hyper-acetylation occurs

at the lysine group directly within both of the nuclear export signals, NLS1 and NLS2, which ultimately results in the extracellular release of HMGB1 (Bonaldi et al., 2003).

Acetylation is not the only post-translational modification of HMGB1 that can have an effect upon its nuclear to cytoplasmic translocation. Phosphorylation has been shown to have a strong influence upon other proteins in regards to their nuclear presence and translocation (Stemmer et al., 2002). This suggests that phosphorylation of HMGB1 may act similar in nature to acetylation of the ligand. However, where acetylation is important for HMGB1 to exit the nucleus, phosphorylation of HMGB1 prevents the molecule from binding to the necessary nuclear import proteins and decreases its overall nuclear presence (Youn and Shin, 2006). HMGB1 phosphorylation may impact the molecule's interaction with nuclear import proteins by affecting key residues (Conti et al., 1998, Stemmer et al., 2002, Stemmer et al., 2003).

The release of HMGB1, and its eventual function as a DAMP, is attributed to cellular insult or injury. First observed and described in macrophages and monocytes, HMGB1 release has been demonstrated in cells outside of the typical immune cell, including neurons both cortical and peripheral in origin (Feldman et al., 2012, Sun et al., 2014, Zou and Crews, 2014). The release of HMGB1 is still being studied and observed, with a focus on vesicle mediated release, from the cytoplasm into the extracellular system (Gardella et al., 2002). The cellular injuries assessed from HMGB1 release include bacterial endotoxin exposure, chemically-induced seizure activity, ischemia-reperfusion injury, and ionic imbalance in the cell media (Zurolo et al., 2011, Feldman et al., 2012). It has been observed that the release of HMGB1 from necrotic cells can also trigger inflammation and

subsequent signal pathways, whereas HMGB1's release during apoptosis does not result in an inflammatory cascade. Given the difference between these two states of cell death, it can be inferred that HMGB1 is used as a means of cellular communication for states of extreme stress and to signal in both an autocrine and paracrine fashion to other cells in order to start necessary recovery processes (Shibasaki et al., 2010, Magna and Pisetsky, 2014).

When HMGB1 is released from the cell of interest, its three key cysteine residues determine the ligand's conformational state and its subsequent receptor affinity. These three cysteine residues are responsible for the particular conformation of the HMGB1 molecule and are affected by the extracellular environment (Yang et al., 2012, Zandarashvili et al., 2013). Upon first release, all three cysteine residues are reduced, and the molecule is in its all-thiol form. When the cysteines at positions 45 and 106 are oxidized, this results in a disulfide bond forming and the disulfide conformation of HMGB1 (Tang et al., 2012a). Further oxidation of the molecule results in the final conformation state, where the HMGB1 is devoid of signaling function and no longer has a mechanism of action (Tang et al., 2012a). This inert form of HMGB1 has been found to be released from apoptotic cells, suggesting that in order to preemptively act against unwarranted inflammation, the apoptotic process must involve neutering the potent signaling capabilities of HMGB1 (Bell et al., 2006, Kazama et al., 2008). As the molecule shifts from the all-thiol state, to containing a disulfide bond, to inert, key binding motifs are revealed. These binding motifs are specific to two different receptors with strong ties to inflammation, the Toll-Like Receptor 4 (TLR4) and the Receptor for Advanced Glycation

End-products (RAGE) (Yang et al., 2013, Lee et al., 2014). In the all-thiol state, HMGB1 has a RAGE binding domain accessible for binding by the receptor from positions 150 to 183, when the molecule is in its all-thiol form after cellular secretion (Yang et al., 2013). The TLR4 binding affinity of HMGB1 (positions 89-108) is only revealed when oxidation at cysteines 23 and 45 result in formation of the disulfide bond (Yang et al., 2013).

Multiple groups have demonstrated that the conformational changes of HMGB1 due to oxidation are reversible and depend upon the extracellular environment to determine its needed role in cellular response to insult or injury. Intriguingly, the half-lives of the all-thiol, disulfide, and inert HMGB1 forms vary greatly in non-pathological or non-injury situations. The all-thiol molecule has a half-life of approximately 17 minutes, with the disulfide form existing in excess of 10 hours, or approximately 642 minutes (Zandarashvili et al., 2013). These values again change when pathology is considered; conditional media used for cell culture of prostate cancer increased the half-life of all-thiol HMGB1, while decreasing that of the disulfide HMGB1 (Zandarashvili et al., 2013). Glycyrrhizin, a licorice root derivative with affinity for HMGB1, significantly increases the half-life of both HMGB1 conformations (Mollica et al., 2007). Interestingly, glycyrrhizin administration has also been demonstrated to have an antagonistic effect on HMGB1 signaling (Mollica et al., 2007, Feldman et al., 2012).

The three identified conformation states of HMGB1 allow the model to compare variably to a skeleton key, with an ability to open multiple locks/bind to different receptors. The aforementioned TLR4 and RAGE have been demonstrated to not only be important to inflammation, but both receptors have affinity to different conformations

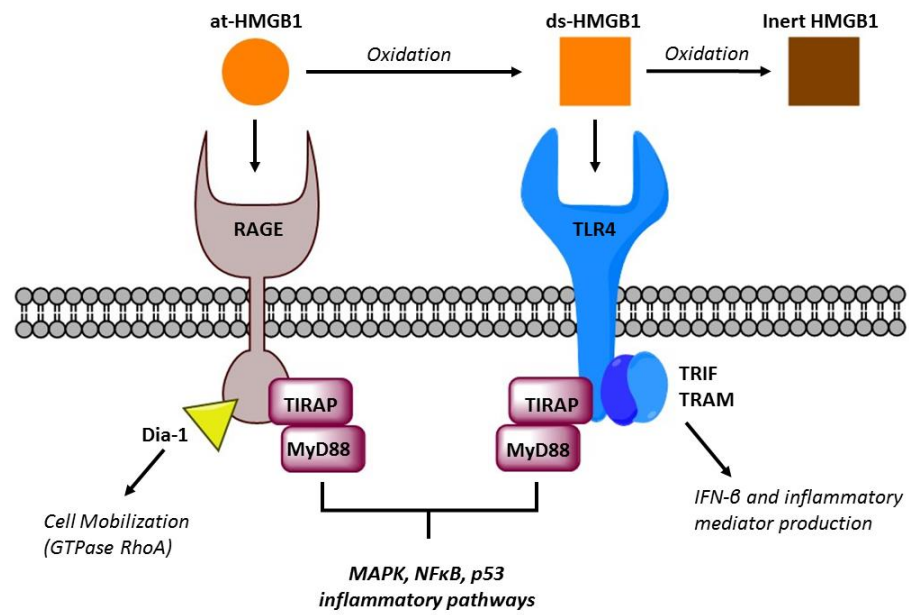
of the HMGB1 molecule. Thus, HMGB1 has at minimum two different receptors and HMGB1's ability to bind to either receptor depends upon the extracellular environment, which can change due to injury, stress, or pathological presence. The different conformations of HMGB1 are mutually exclusive in nature, which by extension means that HMGB1 functions of chemotaxis/cytokine-stimulating and RAGE binding versus TLR4 affinity are also mutually exclusive properties of HMGB1 (Venereau et al., 2012a). In essence, HMGB1, through its ability to help maintain and repair DNA to being capable of initiation of inflammation through various cell types, demonstrates the versatility of the ligand in the aforementioned model of cellular communication. The versatility of HMGB1 becomes more intriguing when framed with HMGB1's functionality in the nervous system, as a factor in multiple disease mechanisms through multiple receptors (Maroso et al., 2010, Shibasaki et al., 2010, Allette et al., 2014).

HMGB1 has been investigated in several formats regarding its inflammatory functionality within the confines of the nervous system. Several publications have demonstrated that HMGB1 has several effects on neuronal tissue, ranging from epileptogenic pathology, to neurodegenerative disorders, traumatic nervous tissue injury, and the focus of this work, pain (Muhammad et al., 2008, Maroso et al., 2010, Shibasaki et al., 2010, Zurolo et al., 2011, Luo et al., 2014, Weber et al., 2014). In each of the aforementioned pathological events, the ligand HMGB1 acts through the receptors TLR4 and RAGE to bring about important developments key to pathology. In order to work towards novel therapeutics broader than the scope of chronic pain, further dissection of the signaling axis HMGB1 shares with TLR4 and RAGE would be incredibly beneficial.

Pain and neuronal pathologies are not the only disease mechanisms that seem dependent upon the function of HMGB1. Increasing evidence in the literature supports the integral role of inflammation in cancer biology (Mantovani et al., 2008). This inflammation presence in cancer and tumor growth has been strongly correlated with significant overexpression of HMGB1 when compared to naïve tissue, exemplified in colon, breast, prostate, and lung cancer (Tang et al., 2010). Post-translation modification of HMGB1 via phosphorylation has been observed to impact the ligand's ability to inhibit or enhance cancer cell replication *in vitro* (Topalova et al., 2008). The ability of HMGB1 to interact with several transcription factors (TFs) is also thought to be important to cancer development. The NF- κ B family of TFs is activated in order to initiate cellular response to various demands, such as growth, apoptosis, homeostasis maintenance, and tumor regulation (Baeuerle and Henkel, 1994). HMGB1 can interact directly with the NF- κ B subunit p50 and affect the subunit's affinity for DNA binding, possibly leading to increased tumor activity (Agresti et al., 2003). The retinoblastoma (RB) protein is another example of HMGB1-mediated cancer development. Improper function of the RB protein can result in a loss of its intrinsic growth suppressive activity and through union with HMGB1; it demonstrates significant increases in cell growth suppression (Jiao et al., 2007). Both the NF- κ B and RB interactions with HMGB1 only show a portion of the role of HMGB1 in the tumor microenvironment (Ellerman et al., 2007). The ligand appears to have a high level of oncological importance, although its exact mechanisms of action are not fully understood or known at this time.

As mentioned earlier, HMGB1 signaling appears to play a significant role in the pathological neuronal hyperactivity seen in epileptogenesis. An epileptic seizure demarcates an event of neurological dysfunction, resulting in abnormal behavior, changes in sensorial perception, and maladaptive neuronal firing (Pitkanen and Lukasiuk, 2009). Epilepsy defines changes in neuronal function that allow and potentially encourage for the recurrence of seizure activity (Pitkanen and Lukasiuk, 2009). One of the subsets of epilepsy, temporal lobe epilepsy, has been observed to involve the HMGB1 in its pathological development (Chiavegato et al., 2014). In a cohort of rats assessed for HMGB1 translocation to the cytoplasm, this translocation was largely confined to the epileptic animals (Choy et al., 2014). Furthermore, glycyrrhizin (known for its affinity for HMGB1 and neutralizing subsequent signaling) was demonstrated in another study to have neuroprotective effects in a rodent model of kainic acid-induced seizures (Luo et al., 2014). In a similar affect to both nociceptive and neuropathic pain, epileptic events involving HMGB1 also demonstrate ties to the receptors RAGE and TLR4, which begets further dissection of the two pattern recognition receptors (Maroso et al., 2010, Zurolo et al., 2011).

Figure 1-1: HMGB1 receptors RAGE and TLR4



The HMGB1 Receptors

TLR4

TLR4 is one of eleven transmembrane receptors that belong to the Toll-like Receptor (TLR) family. These receptors are typically located in the membranes of cells belonging to the innate immune system, including but not limited to, mast cells, monocytes, and macrophages (Barton and Medzhitov, 2002). Cells involved with barrier functionality, such as epithelial and endothelial cells, also express TLR family members (Fitzner et al., 2008, Leow-Dyke et al., 2012). The detection of pathological material and subsequent activation of the necessary inflammatory cascade for the particular insult is one of the main functions of the TLR family (Muzio et al., 1998). Activation of any member of the TLR family results in the secretion of cytokines in order to begin the process of inflammation (Yamamoto et al., 2003). However, as previously stated, and especially in the case of TLR4, the activating ligands for TLRs need not be PAMPs; DAMPs can also serve as endogenous activators of the receptors (O'Neill et al., 2003, O'Neill and Bowie, 2007).

Structurally, all of the members of the TLR family share the Toll IL-Receptor (TIR) domain as part of their respective signaling complexes. In fact, the TIR domain is considered to be representative of an ancestral link between vertebrates and invertebrates in the realm of immunity (Roach et al., 2005). The ligand binding domains of the TLRs are composed of repeating structure motifs rich in leucine; the majority of pattern recognition receptors (including TLRs) either possess the leucine repeating regions, scavenger-receptor protein domains, or calcium-dependent lectin domains

(Medzhitov and Janeway, 1997). Excluding the TLRs 1, 2, and 6, in order for signal propagation after ligand activation, TLRs homodimerize prior to initiation of a signal cascade (Takeda and Akira, 2005). The aforementioned leucine-rich repeating motifs of TLR allow for the dimerization and also allow for the TLR structures to recruit the necessary adaptor proteins within the cell for signal transduction (O'Neill et al., 2003, Takeda and Akira, 2005). Differences between the TLRs occurs with either of their extracellular elements, changing the particular receptor's affinity for specific PAMPs and DAMPs, and adaptor binding proteins attached to the intracellular TIR domain (Hallman et al., 2001).

A commonly-referred agonists of TLR4 is bacterial lipopolysaccharide (LPS). LPS is a major constituent of the outer cellular membrane of gram-negative bacteria and is essential for bacterial survival (Leone et al., 2007). LPS is an exogenous molecule and would be classified as a PAMP, signaling to the host immune system possible infection, resulting in the response of the innate immune system through TLR4 (Nijland et al., 2014). LPS-RS is another exogenous compound that can bind favorably with TLR4; LPS-RS is lipopolysaccharide extracted from *Rhodobacter sphaeroides* and acts as a competitive antagonist to LPS without issuing its own TLR4-mediated signal cascade. However, exogenous molecules such as LPS are considered less as the exclusive ligands for TLR4, thanks in large part due to the waning theory that discernment between self and non-self is of the utmost importance to immune functionality (Erridge, 2010). Endogenous ligands and the concept of 'sterile inflammation' are becoming more prevalent when discussing

TLR4-mediated inflammation. This is exemplified by free fatty acid activation of TLR4, resulting in the production of inflammatory cytokines in adipose tissue (Pal et al., 2012).

In addition, there are several xenobiotic agents that can evoke TLR4 activation, which mandates a level of respect be installed for the promiscuity of the TLR4 receptor. Naloxone and naltrexone are used clinically to initiate partial or complete antagonism of administered opioids, can bind to the TLR4 receptor (Skolnick et al., 2014). Upon binding, either drug can mitigate a subsequent signal cascade, replete with possible mitigation of opioid-induced allodynia (pain from a stimulus that would normally not evoke a pain response) and even the suppression or down-regulation of TLR4 expression (Franchi et al., 2012). Ibudilast, a phosphodiesterase inhibitor, is another example of a pharmaceutical that can be a potential antagonist for TLR4 (Hutchinson et al., 2010). Ibudilast was originally marketed in Japan for the treatment of asthma; research has demonstrated that the drug has anti-inflammatory modalities, theoretically through its TLR4 binding (Rolan et al., 2009). Other xenobiotics that can bind to TLR4 include oxycodone, amitriptyline, fentanyl, and ketotifen (Li, 2012).

Morphine is one of the most commonly used opioids for patient relief in both chronic and severe acute pain settings (1992). Numerous adverse effects aside, it is another example of a xenobiotic agent possessing binding affinity for TLR4 (Stevens et al., 2013). In fact, morphine can demonstrate LPS competitive-inhibition in a concentration dependent fashion, with concentrations as low as 3mM (Madera-Salcedo et al., 2013). This effect of morphine is neither blocked nor augmented in the presence of naloxone (Stevens et al., 2013). Upon metabolism of morphine, morphine-3-glucuronide (M3G) is

produced with no known analgesic activity; M3G also can account for approximately two thirds of the starting pharmacological dose of morphine (Hasselstrom and Sawe, 1993, Wilson et al., 2011). M3G can stimulate the nervous system through activation of TLR4 as its high polarity prevents M3G crossing the blood-brain barrier (Yaksh et al., 1986, Smith, 2000, Due et al., 2012a).

Within the TLR family, TLR4 becomes one of the more complex members due to it possessing the greatest number of intracellular adaptor proteins. After binding of a ligand, TLR4 responds with activation of the core TLR signaling pathways of early-phase Nuclear Factor kappa B (NF- κ B) and Mitogen-Activated Protein Kinase (MAPK). They also can activate the late-phase NF- κ B and Interferon Regulatory Factor-3 (IRF3) pathway, which can lead to further changes in gene expression as well as more specific immune responses, tailored to certain PAMPs or DAMPs (Saitoh et al., 2004). This more diverse group of adaptor proteins theoretically allow TLR4 to modify subsequent signal transduction events to a higher level than the other TLRs. If this is assumed to be correct, the presence of TLR4 on such a diverse range of cell types in addition to a high level of signal modification after activation, allows TLR4 to be an extremely useful component of the cellular response and communication through inflammation (Buchanan et al., 2010).

Prior to activation, the glycoprotein MD-2 can have a significant effect on the levels of TLR4 expression at the cell plasma membrane (Rallabhandi et al., 2008). MD-2 possesses a hydrophobic pocket for ligand binding and forms a multimer complex with a complementing TLR4 homodimer (Kim et al., 2007, Ohto et al., 2007). MD-2 is not only enhances the responsiveness of TLR4 to ligands, specifically LPS; it is actually necessary

for proper LPS-TLR4 binding (Shimazu et al., 1999, Park et al., 2009). In fact, TLR4 knockout mice demonstrate the same lack of response to LPS administration as MD-2 knockout mice when compared to normal activation in naïve animals (Nagai et al., 2002). In human monocytes, MD-2 increases the level of trafficking of TLR4 from the Golgi Apparatus in resting cells to the plasma membrane as demonstrated (McGettrick and O'Neill, 2010).

Upon activation, the TIR domain of TLR4 recruits the requisite adaptor proteins for signal propagation. There are four main TIR domain adaptor proteins, which can be separated by their dependence on the adaptor protein Myeloid Differentiation Factor 88 (MyD88), for their ability to transduce a signal from a ligand effector. The MyD88-dependent pathway involves MyD88, and the Toll-interleukin 1 Receptor domain containing Adaptor Protein (TIRAP). The MyD88-dependent pathway is the core TLR pathway mentioned earlier, functioning to activate the early-phase NF- κ B and MAPK processes (Akira et al., 2006). The MyD88-independent pathway includes the TIR-domain-containing-adaptor-inducing interferon- β (TRIF), and the TRIF-related adaptor molecule (TRAM) (Roach et al., 2005, Akira et al., 2006).

Inflammasome activation is another term to describe the downstream signal cascade TLR4 activates in response to ligand binding. The inflammasome is composed of multiple intracellular proteins within the cell, and acts to connect PAMP recognition by a Pattern Recognition Receptor (PRR), to the eventual maturation and production of the requisite inflammatory factors and cytokines (Lamkanfi and Dixit, 2009, Rathinam and Fitzgerald, 2013). Assembly of the inflammasome occurs in response to extracellular stimuli, such as HMGB1, and takes place in the cytosol of the innate immune cells

(Kayagaki et al., 2013, Keyel, 2014). Altogether, the signaling variances afforded by TLR4 through different ligands, adaptor protein recruitment, and inflammasome formation emphasize their importance in the immune response of an organism, and inflammation. This inflammasome/TLR4 process cannot be attributed to one particular pathway or signal type but rather a component of an intricate network poised to deal with multiple communication needs.

With the functions of TLRs, and TLR4 in particular, to induce inflammatory responses and immune processes, it is curious as to why a neuronal cell would express these receptors, considering that the nervous system is considered immune privileged. Interestingly, TLR4 can affect both neurogenesis and neurodegeneration. Microglial TLR4 function is akin to that of TLR4 on dendritic cells, resulting in increases of both Tumor Necrosis Factor- α (TNF α) and Interferon- β (IFN β) production (Okun et al., 2011). Both microglial and dendritic TLR4 function highlight a primary function of debris analysis and phagocytosis (Okun et al., 2011). Astrocytes, important for biochemical support and homeostasis of the nervous system environment, display low levels of TLR4 expression unless in the presence of inflammation, when their expression increases significantly (Liu et al., 2012, Gong et al., 2014). TLR4 function has also been observed on neuronal cell types, such as neural progenitor cells, cortical and hippocampal neurons, motor neurons, and primary afferent neurons (Bsibsi et al., 2002). The expression level of TLR4 on neurons is dynamic in nature and relies upon the presence of environmental cues, such as DAMP concentrations (McGettrick and O'Neill, 2010).

The expression of TLR4 on sensory neurons in particular has been linked to the processes and development of neuropathic pain (Tanga et al., 2005). A growing body of evidence demonstrates that TLRs, and specifically TLR4, represent a significant presence in the nervous system in both its central and peripheral manifestations (Bsibsi et al., 2002, Acosta and Davies, 2008, Maroso et al., 2010, Okun et al., 2011). Gene knockout mutations for the TLR4 gene in mice results in a significant decrease in both pain behavior and inflammation of the central nervous system (Tanga et al., 2005).

RAGE

The Receptor for Advanced Glycation End-products (RAGE), a member of the immunoglobulin (Ig) superfamily, is a transmembrane protein (Neeper et al., 1992). It is highly conserved across humans, rats, and mice, with expression levels in naïve settings highest in pulmonary tissue, specifically alveolar and pulmonary cells (Sims et al., 2010). RAGE signaling and activation has been linked to several different pathological events, including diabetes, Alzheimer's disease, oncological manifestations, traumatic injury, liver disease, and disorders of chronic inflammation (Bucciarelli et al., 2006, Gebhardt et al., 2008, Chen et al., 2011, Du et al., 2012, Abolfathi Momtaz et al., 2013, Bansal et al., 2013). The signal cascade activated in response to RAGE ligand binding is thought to be an important proponent of tissue repair after insult and a return to environmental homeostasis through inflammatory resolution (Sorci et al., 2012). Baseline levels of RAGE expression are low, RAGE gene expression can significantly increase during pathology (Mangalmurti et al., 2012). The exception to this expression profile being the lungs, where RAGE protein is at high levels on the alveolar epithelium and endothelial cells of the

microvasculature (Mangalmurti et al., 2012). RAGE expressed on the endothelium is able to mediate and affect the adhesion and transmigration of leukocytes through chemoattractant effects (Kierdorf and Fritz, 2013).

Structurally, RAGE is a transmembrane protein and requires the recruitment of adaptor proteins to initiate intracellular signaling cascades (Sorci et al., 2012). The extracellular region of RAGE consists of three Ig domains: a V-type domain integral for ligand specificity and the C1 and C2 domains (Xie et al., 2013). The extracellular region of RAGE is large in size, and a study of the three Ig-like domains (V domain, C1 and C2 domains) reveal that the structure is composed of β -pleated sheets (Bork et al., 1994, Huttunen et al., 1999). The β -pleated sheets contain a disulfide bond, which could impact ligand recognition (Bork et al., 1994, Dattilo et al., 2007). The intracellular domain of RAGE is small in comparison, and demonstrates minimal secondary or tertiary structure, which may affect adaptor protein recruitment for signal transduction (Bork et al., 1994). The structure of the RAGE receptor becomes more interesting with closer inspection of its transmembrane and signaling domains. Across multiple species, RAGE has numerous structural variants due to differential transcript splicing. The RAGE splice variants differ across the cell types and in function. Some variants have no functional capabilities aside from ligand binding, due to changes in the intracellular signaling domain of the RAGE protein, resulting in the inability to recruit the necessary cellular machinery for signal transduction (Sterenczak et al., 2013). These splice variants are referred to as decoy RAGE receptors. Splice variants of RAGE are tissue specific, with certain splices only present in high levels in certain tissue conditions or types (Lopez-Diez et al., 2013).

In addition to splice variants, the metalloproteinase, A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), can cleave the intracellular domain of RAGE from the surface of the cell membrane, producing soluble RAGE (sRAGE) (Sessa et al., 2014). sRAGE can be found in the serum and extracellular milieu, and can act as a decoy or sink for excessive ligands; sRAGE can also be produced through post-translational cleavage processing of the protein (Kalea et al., 2009, Buckley and Ehrhardt, 2010, Lopez-Diez et al., 2013). What is fascinating about this process is that it provides the organism with a fine level of control regarding inflammation. In other words, if an excessive amount of DAMPs are released, not only will the nascent levels of sRAGE or decoy RAGE on cell membranes act to prevent the mitigation of inflammation, these ligand 'heat sinks' can be increased in concentration through ADAM10 up-regulation or post-translational processing of RAGE.

There are multiple ligands that can bind to RAGE, suggesting that RAGE shares the characteristic promiscuity observed in TLR4. Paralleling TLR4, RAGE is also a PPR and plays a key role in the scope of innate responses to PAMPs and DAMPs (Zeng et al., 2012). The promiscuity of the RAGE protein is most likely due to structural design; the C1 and C2 domains of the protein are linked in such a way that allows for free rotation of the extracellular domain of the receptor (Dattilo et al., 2007). This free rotation allows for the combined V and C1 domains to interact with its various signaling compounds through multiple protein surfaces; the rotation does not impact the downstream signal capabilities of the RAGE protein (Dattilo et al., 2007). A significant difference between TLR4 and RAGE is found in their role within the innate immune system. TLR4 is known for

its inflammatory support through the production of cytokines, whereas RAGE acts in the method of cellular migration as demonstrated in an earlier example of leukocyte transmigration to the injured or affected tissue (Schiraldi et al., 2012).

The name RAGE is derived from a large constituent of its specific ligands, advanced glycation end-products (AGE) (Huttunen et al., 1999, Xue et al., 2011). AGEs can be produced as the byproducts of oxidative processes in the extracellular environment (Xue et al., 2011). Chronic inflammatory diseases, diabetic complications, and cardiovascular disorders have all been observed having palpable links to AGE signaling through RAGE (Gebhardt et al., 2008). β -amyloid fibrils are another potential ligand for RAGE. The β -amyloid protein is one of the markers of Alzheimer's disease and demonstrates binding affinity for RAGE, which also increases in concentration when comparing Alzheimer's disease subjects with the appropriate controls (Yan et al., 1996, Yan et al., 2009).

Other possible ligands for RAGE include assorted S100 proteins, nucleic acids (i.e. DNA, RNA), and HMGB1 (Donato, 2007, Luan et al., 2010). The interaction of RAGE and HMGB1 was first investigated in the growth of the nervous system and neurite outgrowth (Hori et al., 1995, Saleh et al., 2013). However, the HMGB1-RAGE axis of inflammatory signaling is also of particular interest given its involvement in several severe pathologies, such as traumatic brain injury and cancer (Okuma et al., 2014, Weber et al., 2014). In the both gastric and colorectal cancers, overexpression of both RAGE and HMGB1 has been shown to be key components of tumor metastasis (Fahmueller et al., 2012). Diabetes is another disease that shows positive correlation with its development and the

upregulation of RAGE, HMGB1, and several key downstream signaling motifs (Mohammad et al., 2012).

RAGE activation can also serve to up-regulate and promote the host's immune defense to microbial agents, through increased in leukocyte activity (Chavakis et al., 2003, van Zoelen et al., 2011). Septic events show increased levels of both mRNA and protein expression of RAGE on the cell plasma membrane. In the murine model, limiting RAGE response in sepsis through genetic or use of antagonists results in an increased survival (Yamamoto et al., 2011). In the case of sepsis, which is considered a LPS-mediated event, there is evidence to suggest that HMGB1 plays a key role in RAGE's inflammatory activity during sepsis (Sunden-Cullberg et al., 2005, Aneja et al., 2008).

The activation of the RAGE receptor initiates activation of NF- κ B and the MAPK signaling cascades. Activation of these cascades through RAGE and its ligands in turn lead to the propagation and perpetuation of inflammation (Kang et al., 2010). The suppression of both NF- κ B and MAPK pathways can be induced by the introduction of neutralizing antibodies for RAGE, or synthesized sRAGE (Lander et al., 1997). Genetic knockout animals for the RAGE gene reveal a diminished susceptibility to not only acute inflammation but tumor-development as well (Gebhardt et al., 2008, Kang et al., 2010).

Another parallel between RAGE and TLR4 can be drawn with regards to their adaptor protein selection. The RAGE signal cascade uses both TIRAP and MyD88 for its eventual inflammatory function, chemoattraction. Diaphenous-1 (Dia1) is another adaptor protein that can bind to RAGE; Dia1 belongs to a family of proteins known as

formins, proteins responsible for proper function and regulation of actin filaments and cytoskeleton formation (Waller and Alberts, 2003). Dia1 acts as an in-between for RAGE and the GTPase RhoA in order to affect cell motility (Waller and Alberts, 2003, Hudson et al., 2008). Downstream of RhoA lies a fork in the signal pathway, with one option being the activation of the N-terminal kinase/activator protein-1 (JNK/AP-1) pathway, the other being the Rac1 and Cdc42 pathway, bringing about the production of chemoattractants, or cell mobilization, respectively (Watanabe et al., 1997, Hudson et al., 2008, Bianchi et al., 2011).

The HMGB1-RAGE signal axis not only shows inflammatory mediator production in several tissue types and disease models; it also demonstrates an intriguing feed-forward mechanism. Specifically, binding of all-thiol HMGB1 to RAGE leads to increased expression of RAGE at the cellular plasma membrane, and increased signal duration through HMGB1 release (Bierhaus et al., 2005). This positive feedback loop is not limited to HMGB1 as a stimuli; advanced glycated end-products (AGEs) result in the increased concentrations of reactive oxygen species (ROS) which in turn act to produce more AGEs (Ott et al., 2014). The feed-forward mechanism of all-thiol and HMGB1 and RAGE provides a focus for study given that chronic inflammation and chronic pain exemplify the results of unmitigated signal transduction. The presence of all-thiol HMGB1 is short lived before it is oxidized into the TLR4 ligand disulfide HMGB1, which suggests that diseases with etiology requiring rapid and uncontrolled inflammatory response may look to the HMGB1-RAGE axis for further insight.

Expression of RAGE in the nervous system is not limited to any particular cell type. In positive correlation with TLR4, RAGE has also been observed within the nervous system environment both on neurons and glial cells (Schmidt et al., 2001, Miller et al., 2008, Iori et al., 2013). Currently studies suggest the presence of RAGE on neuronal membranes, in conjunction with its activation, may provide a front line defense mechanism against neurotoxic levels of inflammation. However, RAGE has also been shown to be tightly connected with the development and sometimes fatal propagation of assorted neurodegenerative disorders (Sousa et al., 2001). This convoluted nature and role of RAGE in the neuronal setting may simply be a result of differential downstream signaling, but also the initiating ligand responsible for RAGE activation (Kierdorf and Fritz, 2013). Neural regeneration is another area of RAGE action within the peripheral nervous system. Specifically the absence of the cell surface RAGE receptor has demonstrated a lowered level of signaling after neuronal injury, decreased neurite outgrowth, and a reduced amount of monocyte infiltration (Rong et al., 2004b).

In the instance of RAGE and HMGB1 signaling, evidence has linked the interaction of these two proteins in a capacity of growth, specifically neurite outgrowth (Huttunen et al., 1999). Specifically, the RAGE pathway utilizing the activation of Rac and Cdc42 was linked to the process of neurite outgrowth (Huttunen et al., 1999). Introduction of HMGB1 into the sciatic nerve results in promotion of immune activation, while simultaneously resulting in pain hypersensitivity (Shibasaki et al., 2010). Ligation of the same sciatic nerve shows a significant increase in the expression of RAGE as detected by immunofluorescence in the damaged nerve and the majority of neurons in the associated

dorsal root ganglia (Shibasaki et al., 2010). Antagonism of HMGB1 binding and activation resulted in a decrease in both pain hypersensitivity as well as mechanical allodynia (Shibasaki et al., 2010). Since RAGE, TLR4, and HMGB1 are major factors in the inflammatory process, and they are expressed in the nervous system, further investigation into their signal cascades, the adaptor proteins, is of importance.

The TIR Domain

Both TLR4 and RAGE share the adaptor proteins TIRAP and MyD88 as part of their respective signal cascades (Horng et al., 2001, Sakaguchi et al., 2011). Since both receptors are important in innate immunity and have been linked to severe pathogenic consequences, dissection of the receptors' functionality may prove most efficacious when focused upon the adaptor proteins (Ibrahim et al., 2013). TIRAP and MyD88 make up the MyD88-dependent signaling pathway of both RAGE and TLR4, with TRAM and TRIF being exclusive to TLR4 function and MyD88-independent in signaling activity (Yamamoto et al., 2003, Ibrahim et al., 2013).

As stated previously, TIRAP and MyD88 bind to the TIR domain of the recruiting receptor to transduce the related signal of host defense or inflammation, according to the receptor-bound ligand. To be more specific, TIRAP, which may also be known as MAL (MyD88-Adaptor-Like), functions by binding to the receptor and then recruiting MyD88 afterwards (Yamamoto et al., 2004, O'Neill and Bowie, 2007). This was demonstrated by the attenuation of TLR signaling through genetic manipulation of TIRAP; this mutated TIRAP also resulted in a significant decrease in recruited MyD88 to the cellular membrane

(George et al., 2010). TIRAP in particular is highly conserved, suggesting a standard role within host defense across species, including humans, mice, and zebrafish (Woo, 2012).

Extracellular domains and differential ligand binding affinities aside, it is the recruitment and function of the adaptor proteins that bring about the specific characteristics of both RAGE and TLR4 that are so heavily studied. This shared adaptor protein aspect between RAGE and TLR4 suggests possible crosstalk between receptors and may further explain their separate but related roles in the mitigation of neuronal excitation. Additionally, targeting the adaptor proteins TIRAP and MyD88 may provide an interesting model for novel treatment of multiple disease pathologies, including those specifically of chronic inflammation such as chronic pain. If the loss-of-function genetic manipulations can result in the decrease of signaling functionality for both or either TLR4 and RAGE, then pharmacological targeting with small molecule inhibition may prove highly effective in a therapeutic manner (Khor et al., 2007, Nejentsev et al., 2008). Since inflammation is a process key to survival, the benefit of utilizing pharmacology is dosing; activation of HMGB1 does not need to be attenuated, but rather controlled in order to prevent pathological events.

Thesis Aims

The ultimate goal of this research project was to reveal the key mechanisms and components of the HMGB-1-dependent activation states that are present during hyperactivity and excitation of both nociceptive and non-nociceptive sensory neurons. By delving into this topic, the results of this research may lend themselves to uncovering

novel therapeutic targets for maladies deriving from chronic inflammatory signaling. Further investigation may also elucidate and provide a better understanding of the mechanisms of inflammation that are present in the nervous system, primarily that within the peripheral circuit.

Within this dissertation, I will investigate both conformation states of HMGB1, all-thiol and disulfide, as well as the expression and function of both TLR4 and RAGE. A large focus will be also placed upon the role of both TIRAP and MyD88 as the associated adaptor proteins associated with both the HMGB1-TLR4 and HMGB1-RAGE signal cascades. If the common pathway between RAGE and TLR4 is evident, pharmacological targeting of the adaptor protein complex could serve as suitable targets for the control of the inflammation to prevent pathological development within the organism.

The first specific aim of this project is to **determine the degree to which the all-thiol HMGB1 and disulfide HMGB1 conformations are integral to both RAGE and TLR4 nociceptive neuronal signaling.** Given the alarmin's aforementioned flexibility with receptor-mediated binding and overall mobility within the intracellular and extracellular spaces, HMGB1 is an ideal target of study. Previous work in the White lab has noted that HMGB1 is released in states of neuronal cellular stress and mediates observed pain behavior in a peripheral nerve injury model (Feldman et al., 2012). This data, in combination with findings of HMGB1 organically converting from all-thiol (RAGE) to disulfide (TLR4) forms, implicates HMGB1 as being the key connection between both RAGE and TLR4 being observed as integral to pain mediation via neuronal excitation (Zandarashvili et al., 2013).

The second aim of this thesis is to **investigate the dependency of neuronal excitation upon functionality of the MyD88 and TIRAP adaptor proteins following ligand binding and activation of both RAGE and TLR4**. Both RAGE and TLR4 are linked to the MyD88 and TIRAP adaptor proteins, thus these adaptor proteins are most likely highly integral to proper signaling cascades for both receptors. In turn, this signifies the importance of both adaptor proteins to neuronal excitation. The forthcoming assays should reveal both MyD88 and TIRAP as key targets for modulation of chronic neuronal inflammation.

Chapter 2: HMGB1 and Nociceptive Neuronal Signaling

Identification of a functional interaction of HMGB1 with Receptor for Advanced Glycation End-products in a model of neuropathic pain

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Abstract

Recent studies indicate that the release of high mobility group box 1 (HMGB1) following nerve injury may play a central role in the pathogenesis of neuropathic pain. HMGB1 is known to influence cellular responses within the nervous system via two distinct receptor families; the Receptor for Advanced Glycation End-products (RAGE) and Toll-like receptors (TLRs). The degree to which HMGB1 activates a receptor is thought to be dependent upon the oxidative state of the ligand, resulting in the functional isoforms of all-thiol HMGB1 (at-HMGB1) acting through RAGE, and disulfide HMGB1 (ds-HMGB1) interacting with TLR4. Though it is known that dorsal root ganglia (DRG) sensory neurons exposed to HMGB1 and TLR4 agonists can influence excitation, the degree to which at-HMGB1 signaling through neuronal RAGE contributes to neuropathic pain is unknown. Here we demonstrate that at-HMGB1 activation of nociceptive neurons is dependent on RAGE and not TLR4. To distinguish the possible role of RAGE on neuropathic pain, we characterized the changes in RAGE mRNA expression up to one month after tibial nerve injury (TNI). RAGE mRNA expression in lumbar dorsal root ganglion (DRG) is substantially increased by post-injury day (Holmbeck et al.) 28 when compared with sham injured rodents. Protein expression at PID28 confirms this injury-induced event in the DRG. Moreover, a single exposure to monoclonal antibody to RAGE (RAGE Ab) failed to abrogate pain behavior at PID 7, 14 and 21. However, RAGE ab administration produced reversal of mechanical hyperalgesia on PID28. Thus, at-HMGB1 activation through RAGE may be responsible for sensory neuron sensitization and mechanical hyperalgesia associated with chronic neuropathic pain states.

Introduction

Recent studies indicate *inflammatory* mediators released by nerve injury play a central role in the pathogenesis of *chronic pain conditions* (Calvo et al., 2012). Although poorly understood, a key feature of these inflammatory events is the presence of Danger Associated Molecular Patterns (DAMPs; alarmins) (Bianchi, 2007). A DAMP of particular interest to the injured nervous system is high mobility group protein box-1 (HMGB1; previously known as amphoterin) (Andersson and Tracey, 2011). HMGB1, a nuclear protein that binds DNA and regulates gene expression is structurally composed of two tandem DNA-binding domains, Box A and B, and a highly acidic C-terminal tail composed of a string of aspartate and glutamate residues (Bianchi et al., 1992, Giese et al., 1992, Czura et al., 2001, Dumitriu et al., 2005). Originally described as a membrane-associated protein that regulated neurite outgrowth during development, it is now known that HMGB1 also plays a crucial role in the inflammatory responses associated with tissue injury, reparative responses and disease (Parkkinen et al., 1993, Hori et al., 1995, Ulloa and Tracey, 2005, Maroso et al., 2010, Zhang et al., 2011) and may contribute significantly to chronic neuropathic pain states (Shibasaki et al., 2010, Feldman et al., 2012).

The action of HMGB1 on different cell types is known to differ dramatically based on the oxidation state of the protein. When first released into the extracellular space, HMGB1 is initially in the all-thiol state (at-HMGB1) and is thought to largely act on a member of the Ig superfamily, the Receptor for Advanced Glycation End-products (RAGE) (Huttunen et al., 2002). There are also reports that at-HMGB1 can form a complex with CXCL12 and act through CXCR4 (Venereau et al., 2012b). Once present in an oxidative

environment, cysteines 23 and 46 of HMGB1 Box A form a sulfide bond, effectively producing the disulfide isoform of HMGB1 (ds-HMGB1). ds-HMGB1 appears to primarily act on the receptor toll-like receptor 4 (TLR4) in order to influence the production of inflammatory cytokines (Venereau et al., 2012b, Yang et al., 2012). ds-HMGB1 can then be further reduced by sulfonation of cysteine 106 in the Box B domain of the ligand, resulting in an inert form (Kazama et al., 2008).

It has been suggested that release of HMGB1 from injured neurons can contribute to seizure activity associated with epilepsy; however, the receptor responsible for this pathological activity is still disputed. (Maroso et al., 2010, Iori et al., 2013). The actions of HMGB1 have also been implicated in both inflammatory and neuropathic pain conditions, though it is unclear as to whether the TLR4 or RAGE receptor is responsible (Chacur et al., 2001, O'Connor et al., 2003, Shibasaki et al., 2010, Otoshi et al., 2011, Feldman et al., 2012, Nakamura et al., 2013). Endotoxin-mediated TLR4 activation is known to directly increase neuronal excitation states in acutely dissociated nociceptive neurons (Hua et al., 1996, Ochoa-Cortes et al., 2010, Diogenes et al., 2011b, Due et al., 2012b) and administration of xenobiotic TLR4 agonists can produce tactile behavioral hypersensitivity in uninjured rodents (Due et al., 2012b). Since the effects of HMGB1 could be mediated by either TLR4 and RAGE depending on the oxidation state of the protein, we set out to determine the contribution of at-HMGB1 on neuronal excitation of nociceptive neurons using a small molecule inhibitor of TLR4 (Bevan et al., 2010) and a neutralizing antibody against RAGE, 11E6 (Guo et al., 2012, Strakhova and Desiree-Brderson, 2013). Moreover, as little direct evidence exists to support a direct role for RAGE in pain, additional studies

examined tibial nerve injury (TNI)-induced RAGE expression in associated lumbar DRG and the ability of RAGE neutralizing antibody to reverse injury-induced behavioral hypersensitivity in the rat across time. Our data indicates that at-HMGB1 elicits neuronal excitation via RAGE, in acutely dissociated sensory neurons. In addition, the use of the neutralizing RAGE antibody reverses tactile pain hypersensitivity. This evidence, together with the increased expression of RAGE in the sensory ganglia, identifies a new potential therapeutic target which appears to contribute to pathological pain.

Methods

Animals

Pathogen-free, adult female and male Sprague-Dawley (S/D) rats (150–200 g; Harlan Laboratories, Madison, WI) were housed in temperature ($23 \pm 3^{\circ}\text{C}$) and light (12-h light: 12-h dark cycle; lights on at 07:00 h) controlled rooms with standard rodent chow and autoclaved tap water available. Experiments were performed during the light cycle. Animals were randomly assigned to the treatment groups. All animal related experiments were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines established by the International Association for the Study of Pain.

Tibial Nerve Injury

All rodents were anesthetized during the procedure with isoflurane (4% induction, 2% maintenance). To model neuropathic pain, we performed a tibial nerve injury (TNI) [23; 30; 58]. S/D rats 150-200g were anesthetized using isoflurane at 4% induction and 2% maintenance. Under anesthesia, the right sciatic nerve was isolated under aseptic surgical conditions by blunt dissection of the femoral biceps muscle, without damaging the epimysium. The sciatic nerve and its three branches were isolated: the sural, common peroneal and tibial nerves; only the tibial nerve was tightly-ligated with 5-0 silk and transected distal to the ligation. The removal of an additional 2-4mm of distal nerve stump was removed in order to prevent re-innervation by the proximal end of the nerve.

The overlying muscle and skin was then sutured in two separate layers. Sham-injured animals were subjected to all preceding procedures with the exception of ligation and transection.

Reagents

All reagents were freshly prepared in buffer on day of use. The neutralizing monoclonal RAGE antibody (11E6) was generated against the murine C2-domain of RAGE (Guo et al., 2012, Strakhova and Desiree-Brderson, 2013) (Abbvie Laboratories, Deerfield, IL). Administration of 11E6 or control (Ig1 non-neutralizing) antibody was given as intraperitoneal injections. A TLR2 [(Cheng et al., 2012); CU-CPT22] and a TLR4 [(Bevan et al., 2010); compound 15] small molecule inhibitor were synthesized as previously described (Kindly provided by Hang Yin, University of Colorado). LPS was purchased by Sigma (St. Louis, MO). Non-oxidizable, chemotaxis-HMGB1 (all-thiol) and cytokine-HMGB1 (disulfide) was purchased from HMGBiotech (Milan, Italy; <1.0 endotoxin per 1 g of the protein by the LAL method), and was reconstituted in sterile 0.1% BSA/PBS.

Behavioral assessment

All rodents were habituated to testing chambers for at least two days. Rodents were randomly assigned to sham or injured test groups. All baseline testing occurred before and after TNI. The incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hindpaw was measured with a flat-tipped cylindrical probe (Von Frey filament) measuring 200 μ m in diameter [6; 34]. Von Frey filaments capable of exerting forces of 10, 20, 40, 60, 80 and 120 mN with a uniform tip diameter was applied to a designated loci present on the plantar surface of the foot.

During each test, the rodent was placed in a transparent plastic cage with a wire grid floor with ~1°-1 cm openings. The cage was elevated so that stimulation was applied to each hind foot from beneath the rodent. The filaments were applied in order of ascending force, and each filament was applied alternately to each foot. The duration of each stimulus was approximately 1 s and the inter-stimulus interval was approximately 10–15 s. The incidence of foot withdrawal was expressed as a percentage of the 6 applications of each stimulus and the percentage of withdrawals was then plotted as a function of force. The von Frey withdrawal threshold was defined as the force that evoked a minimum detectable withdrawal observed on 50% of the tests given at the same force level. For cases in which none of the specific filaments used evoked withdrawals on exactly 50% of the tests, linear interpolation was used to define the threshold. Pre-TNI baseline behavioral assessment was established in all rodents. The rats were tested pre dose and 1 h post dose on days 7, 14, 21 and 28. Optimum 11E6 dosing was established using TNI animals at day 28 using 1, 5, 10, and 15 mg/kg (data not shown). For drug studies, all behavioral assessments were performed by blinded pain assessors.

RNA isolation and RT-qPCR

Lumbar dorsal root ganglia (DRG; L4-L5) were dissected from adult female Sprague Dawley rats, frozen in liquid nitrogen, and maintained at -80°C until processed for RNA extraction. Total RNA was extracted from the samples using the RNeasy RNA extraction and purification kit (Qiagen). Single stranded cDNA was synthesized using reverse transcriptase (Bioline) with oligo-dT primers. Quantitative PCR was performed as previously described [10]. Briefly, resultant cDNA samples were amplified on an ABI

PRISM 7900HT Sequence Detection System (Applied Biosystems) using the reporter, SYBR Green. The PCR reaction was as follows: 1x, 50°C, 2 min; 1x, 95°C, 10 min; 45x, 95°C, 15 s, 60°C, 1 min; 1x, 25°C, hold. To check for DNA contamination, PCR was run using an *L27* (ribosomal housekeeping gene) primer pair, whose PCR product crosses an intron. The mRNA level for each gene (x) relative to *L27* mRNA (internal control) was calculated using the following equation where Ct refers to threshold cycles: $\text{mRNA (x\%)} = 2^{\text{Ct (L27)} - \text{Ct(x)}} \times 100$.

Western blot analysis

Female animals were sacrificed and transcardially-perfused with saline and tissue was removed and frozen immediately with liquid nitrogen and stored at -80°C. The fresh frozen L4-L5 DRG tissue samples, ipsilateral to the injury, were homogenized in RIPA buffer with protease/phosphatase inhibitors and protein concentration was determined using the BCA protein assay (Thermoscientific). Samples (40 µg/lane) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After incubation in 10% non-fat milk blocking solution overnight at 4°C, the membrane was incubated with rabbit anti-RAGE (1:1,000; Sigma–Aldrich) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). The membrane was probed again with a monoclonal anti- β actin antibody (1:5,000; Sigma– Aldrich, St. Louis, MO). Immunopositive bands were detected by enhanced chemiluminescence (ECL) and measured by a densitometric analysis (UnscanIt; Silk Scientific Inc., Orem, UT, USA).

Preparation of acutely dissociated dorsal root ganglion neurons

The L₄-L₆ DRGs were acutely dissociated using methods described by Ma and LaMotte (Ma and LaMotte, 2005). Briefly, L₄-L₆ DRGs were removed from uninjured female animals. The DRGs were treated with collagenase A and collagenase D in HBSS for 20 min (1 mg/ml; Roche Applied Science, Indianapolis, IN), followed by treatment with papain (30 U/ml, Worthington Biochemical, Lakewood, NJ) in HBSS containing 0.5 mM EDTA and cysteine at 35 C. The cells were then dissociated by mechanical trituration in culture media containing 1 mg/ml bovine serum albumin and trypsin inhibitor (Worthington Biochemical, Lakewood, NJ). The culture media was Ham's F-12 mixture, DMEM, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 µg/ml and 100 U/ml) and N2 (Life Technologies). The cells were then plated on coverslips coated with poly-L lysine and laminin (BD bioscience) and incubated for 2–3 h before more culture media was added to the wells. The cells were then allowed to sit undisturbed for 12–15 h to adhere at 37° C (with 5% CO₂).

Following some *in vitro* experiments, cells were fixed for 10 minutes using 2% buffered paraformaldehyde, washed with saline and incubated with blocking buffer (3% BSA/3% horse serum/0.4% Triton-X; Fisher Scientific, Pittsburgh PA) for 1 hour, followed by overnight incubation with the goat polyclonal antisera generated against TLR4 L14 extracellular monoclonal antibody (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-RAGE (1:1,000; Sigma–Aldrich) at 4° C. Additional monoclonal antibodies were used against CGRP (1;100; Rockland Inc) and IB4 conjugated directly to fluorescein isothiocyanate (*IB4-FITC; Sigma-Aldrich*). After primary incubation, secondary antibodies (anti-rabbit, anti-goat or anti-mouse conjugated to CY3 and CY2,

made in donkey at 1:800; Jackson ImmunoResearch, West Grove, PA) were used to visualize cells. Coverslips were washed in PBS for 5 min each (×3), stained with Hoechst 33258 nuclear marker (Invitrogen Corporation, Carlsbad CA) and mounted with a PBS/glycerol solution onto glass slides.

Ca²⁺ imaging

The dissociated DRG cells were loaded with fura-2 AM (3 mM, Invitrogen Corp., Carlsbad, CA USA) for 25 minutes at room temperature in a balanced sterile salt solution (BSS) (NaCl (140 mM), Hepes (10 mM), CaCl₂ (2 mM), MgCl₂ (1 mM), glucose (10 mM), KCl (5 mM). The cells were rinsed with the BSS and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera coupled to a microscope and MetaFluor software (Molecular Devices Corp., Downingtown, PA USA). Cells were illuminated with a 150 W xenon arc lamp, and the excitation wavelengths of the fura-2 (340/380 nm) were selected by a filter changer. Sterile solution was applied to cells prior to HMGB1 application, any cells that responded to buffer alone were not used in neuronal responsive counts. HMGB1 (27 μM) was applied directly into the coverslip bathing solution. HMGB1 was purchased from R&D Systems (Minneapolis, MN, USA; <1.0 endotoxin per 1 g of the protein by the LAL method), and was reconstituted in sterile 0.1% BSA/PBS. 27 μM of HMGB1 was applied for calcium imaging. A period of three minutes was allowed for observation after the treatment was introduced to the bath. After HMGB1 application, LPS (1μg/mL) and capsaicin (3 nM) were added. Calcium imaging

traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts.

Electrophysiology

Sharp-electrode intracellular recordings were obtained from primary afferent neurons 12-18 hours after dissociation. Coverslips were transferred to a recording chamber that was mounted on the stage of an inverted microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, NY). The chamber was perfused with a bath solution containing (mM): NaCl 120, KCl 3, CaCl₂ 1, MgCl₂ 1, Hepes 10, Glucose 10, adjusted to pH 7.4 and osmolarity 300 mosM. The recordings were obtained at room temperature. Intracellular recording electrodes were fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL) and pulled on a Flaming/Brown micropipette puller (P-98, Sutter Instruments, Novato, CA). Electrodes were filled with 1.0 M KCl (impedance: 40-80 M Ω) and positioned by a micromanipulator (Newport Corporation, Irvine, CA). -0.1 nA current injection was used to bridge-balance the electrode resistance. Diameter was measured by visual examination prior to electrode impalement using a calibrated eyepiece graticule within the microscope. The size of the soma to be recorded was classified according to its diameter as small ($\leq 30 \mu\text{m}$), medium (31–45 μm) and large ($\geq 45 \mu\text{m}$). Electrophysiological recordings were performed with continuous current-clamp in bridge mode using an AxoClamp-2B amplifier, stored digitally via Digidata 1322A interface, and analyzed offline with pClamp 9 software (Axon Instruments, Union City, CA). A neuron was accepted for study only when it exhibited a resting membrane potential (Balermipas et al.) more negative than -45 mV. For each

neuron isolated for study, a continuous recording was obtained for 1 minute without the delivery of any external stimulus. Neuronal excitability of small and medium diameter dissociated DRG sensory neurons was measured by injecting 1-s current pulses into the soma every 30 s. Current was adjusted in order to elicit 1-2 action potentials per current injection under baseline conditions. Non-responding cells were discarded. Following 3 control current injections, at-HMGB1 (27 μ M) or LPS (1 μ g/mL) was applied to the coverslip and current injections continued every 30 s. Neuronal excitability was measured as number of action potentials elicited per current pulse before and immediately after addition of HMGB1 (15 and 45 s, respectively). If at-HMGB1 increased neuronal excitability, RAGE 11E6 (50 μ M), TLR4 small molecule inhibitor (compound 15, 50 μ M), or TLR2 inhibitor (CU-CPT22, 50 μ M) was added to the bath to determine if at-HMGB1-elicited neuronal excitation could be reversed. If LPS increased neuronal excitability, RAGE 11E6 was added to the bath to determine if LPS-elicited neuronal excitation could be reversed. Excitation and blocking of excitation was analyzed within the first two minutes of application of RAGE 11E6, TLR2 or TLR4 inhibitors.

Statistics

GraphPad Software (LaJolla, CA) was used to determine the statistical significance. Results were expressed as mean \pm SEM. When only two groups were compared, Student's unpaired t test was used. Multiple comparisons were evaluated by Dunnett's multiple comparison test after one-way ANOVA. $p < 0.05$ was considered to be statistically significant. GraphPad Software was used to determine the statistical significance of

differences in calcium response among HMGB1 oxidation state groups using Chi-square test with Yates correction with $p < 0.05$ set as statistical significance.

Results

Neuronal RAGE-immunoreactivity colocalizes with TLR4, the isolectin IB4 and calcitonin-gene related peptide (CGRP) expression.

We previously reported that the TLR4 receptor is localized to both peptidergic and non-peptidergic sensory neurons within the dorsal root ganglia (Due et al., 2012b). We examined cells *in vitro* for co-expression of RAGE and TLR4, IB4 and CGRP. Dissociated L₄ and L₅ DRGs removed from naïve animals and cultured for 16-20 hours, exhibited a near complete colocalization of TLR4 and RAGE in small and medium diameter neurons (**Fig. 1 B, C**) with little to no TLR4 or RAGE immunoreactivity present in non-neuronal cells (**Fig. 1 A**). Further examination revealed that there was some colocalization of the RAGE with the non-peptidergic marker of nociceptive neurons, IB4 (**Fig. 1 E, F**), and the peptidergic nociceptive neuronal marker, CGRP (**Fig. 1 H, I**).

Differential increases in neuronal $[Ca^{2+}]_i$ by oxidation state specific HMGB1

To determine whether neuronal RAGE or TLR4 receptors are capable of modulating depolarization-evoked Ca^{2+} transients, we used ratiometric Ca^{2+} imaging to measure at-HMGB1 or ds-HMGB1 responses in neurons isolated from rat lumbar DRG. As many LPS-sensitive sensory neurons are activated by capsaicin (Due et al., 2012b), we also compared the actions of at-HMGB1 and ds-HMGB1 in LPS-sensitive nociceptive and non-nociceptive sensory neurons. Virtually all responsive neurons were small ($\leq 30 \mu m$) to medium diameter cells (31-45 μm). Exposure to media containing at- or ds-HMGB1 at a 27 μM concentration for 3 minutes was used as a maximal stimulus. This treatment produced a total of 14 of 96 cells that were responsive to at-HMGB1, of which only 6 cells

responded to both at-HMGB1 and LPS (**Table 1**). Upwards of 70.1% of at-HMGB1 responsive cells were nociceptive (capsaicin-sensitive) (**Table 2**). These data indicate that many at-HMGB1 responses *in vitro* were likely mediated by the RAGE receptor.

When sensory neurons were subjected to ds-HMGB1, considerably fewer cells exhibited calcium transients (**7.55%**; **Table 1**). Though there was a decrease in the percentage of total responsive cells, 100% of the disulfide HMGB1-responsive neurons responded to LPS (**Table 1**) and only 42% of the assayed cells were sensitive to capsaicin (**Table 2**). Together, these results support the functional expression of both RAGE and TLR4 in nociceptive and non-nociceptive DRG neurons *in vitro*.

All-thiol HMGB1-elicited increase in sensory neuron excitation is dependent on RAGE and not TLR4

HMGB1-induced neuronal hyperexcitability has been recently described in rodent models of cerebral ischemia, epilepsy (Maroso et al., 2010, Qiu et al., 2010) and in DRG sensory neurons *in vitro* (Feldman et al., 2012). Though it is now known that oxidation state-dependent HMGB1 interactions signal through specific receptor types (RAGE receptor or TLR4 receptor), it is largely unknown what receptor is responsible for neuronal excitation (Yang et al., 2010, Venereau et al., 2012b, Yang et al., 2012). To determine the identity of the functional receptor by which oxidation state specific forms of HMGB1 can induce changes in sensory neuron excitability, we examined cellular responses using current clamp in the presence of at-HMGB1. If the cells responded to at-HMGB1, a small molecule inhibitor for TLR4 and TLR2, or the RAGE 11E6 antibody was administered followed by repeated current pulses. Due to the limited numbers of cells that were

responsive to ds-HMGB1 (see **Table 1**), we substituted this form of HMGB1 for the known agonist of TLR4, LPS. Administration of the toll receptor antagonists or RAGE 11E6 alone failed to alter resting membrane potentials (data not shown).

Repeated current pulses in the presence of at-HMGB1 produced a significant increase in the excitability of small diameter sensory neurons when compared to baseline levels (1.4 ± 0.2 action potentials (APs) for control vs. 5.7 ± 0.4 APs for at-HMGB1) (**Fig. 2 A**). Bath exposure to at-HMGB1 in combination with the TLR4 small molecule inhibitor (compound 15) and repeated current pulses failed to alter repeated current pulse induced APs (5.1 ± 0.4 APs; $n=5$; ANOVA, interaction $F(3,14) = 52.60$, $p < 0.0001$; Bonferroni's multiple comparisons, $p > 0.05$ for at-HMGB1 vs. at-HMGB1 + Comp.15) (**Fig. 2 A, B**). Following exposure to at- HMGB1 plus RAGE antibody 11E6, we observed a statistically significant decrease in the number of APs when compared with at-HMGB1 alone (1.2 ± 0.1 APs for control vs. 6.8 ± 1.3 APs for at-HMGB1 vs. 1.6 ± 0.2 APs for at-HMGB1 + RAGE Ab; $n=5$; ANOVA, interaction $F(3,14) = 17.36$, $p < 0.001$; Bonferroni's multiple comparisons, $p < 0.001$ for at-HMGB1 vs. at-HMGB1 + RAGE antibody, 11E6) (**Fig 2. C, D**). The consequences of at-HMGB1 neuronal exposure in the presence of TLR2 inhibitor (CU-CPT22) were similar to those observed with Compound 15 (1.1 ± 0.1 APs for control vs. 4.3 ± 0.4 APs for at-HMGB1 vs. 3.7 ± 0.2 APs for at-HMGB1 + CU-CPT22; $n=5$; ANOVA, interaction $F(3,14) = 32.43$, $p < 0.0001$; Bonferroni's multiple comparisons, $p > 0.05$ for at-HMGB1 versus at-HMGB1 + CU-CPT22) (**Fig. 2 E, F**).

LPS-elicited increase in sensory neuron excitation is not dependent on RAGE

Given the ability of LPS to elicit an inward current in sensory neurons (Ochoa-Cortes et al., 2010, Diogenes et al., 2011b, Due et al., 2012b), we also tested whether the LPS-induced neuronal responses were dependent on TLR4 or RAGE. Representative recordings and grouped data demonstrate that the excitability of sensory neurons was significantly increased by LPS when compared with control levels (**Fig. 2 G, I**). We then utilized the RAGE 11E6 antibody to discern the degree to which LPS-induced neuron response was dependent on the RAGE receptor. RAGE 11E6 did not alter endotoxin-induced APs (1.2 ± 0.1 APs for control vs. 4.3 ± 0.4 APs for LPS vs. 4.9 ± 0.4 APs for LPS + RAGE 11E6; $n=5$; ANOVA, interaction $F(3,11) = 32.65$, $p < 0.0001$; Bonferroni's multiple comparisons, $p > 0.05$ for LPS vs. LPS + RAGE 11E6) (**Fig. 2 G, H**).

A previously reported small molecule inhibitor of TLR4, Compound 15, was then used as a chemical probe to further investigate the molecular mechanism of LPS-induced neuron response (Bevan et al., 2010, Due et al., 2012b). Similar to our previous observations, Compound 15 completely blocked the increased excitability of LPS (1.2 ± 0.1 APs for control vs. 4.9 ± 0.8 APs for LPS vs. 1.7 ± 0.2 APs for LPS + Comp. 15; $n=5$; ANOVA, interaction $F(3,14) = 29.95$, $p < 0.0001$; Bonferroni's multiple comparisons, $p < 0.001$ for LPS vs. LPS + Comp. 15) (**Fig. 2 I, J**). In contrast, Compound 15 demonstrated no effect upon blocking the increased neuronal hyperexcitability induced by HMGB1 treatment (**Fig. 2 A, B**).

RAGE expression in the Lumbar DRG following Tibial Nerve Injury

Previous findings suggest that HMGB1 may be important for neuropathic pain behavior in rodents (Shibasaki et al., 2010) and that HMGB1 can be released for extended

periods of time following peripheral nerve injury (Feldman et al., 2012). Based on the kinetic breakdown of at-HMGB1 in different oxidative environments, it is likely that the neuronal RAGE receptor may be associated with nociceptive behavior in the rodent. In order to better understand the potential influence of RAGE in rodent neuropathic pain models, we studied changes in expression of RAGE and other genes known to be modified following peripheral nerve injury including the $\alpha 2\delta 1$ auxiliary subunit of the voltage-dependent calcium channel (CACNA2D1) and the voltage-gated sodium channel isoform 1.8 (Nav1.8) SCN10A. We initially quantified the mRNA level of individual genes in the DRG using quantitative real time PCR. Although CACNA2D1 mRNA appeared to increase from naïve levels to post-injury day 14 and 28, the data was not significantly different (**Fig. 3A; n=4**, ANOVA, $F = 2.51$; $p > 0.05$). SCN10A mRNA level comparing naïve control tissue with post-injury day 14 and 28 also lacked a statistically different level of significance (**Fig. 3B; n=4**, ANOVA, $F = 0.62$; $p > 0.05$). RAGE expression was strongly upregulated by PID 28 when compared to both PID 14 and naïve control tissue (**Fig. 3C; n=4**, ANOVA, $F = 8.41$; $p < 0.05$). Given the pronounced effect of TNI on RAGE mRNA expression within the L4-L6 DRGs at PID28, we next compared the TNI-induced changes on RAGE protein expression levels in L4-L6 DRGs. Lumbar DRG derived from TNI rodents exhibited a 3-fold increase in RAGE protein levels at PID28 (**Fig. 4; n=6**, ANOVA, $F = 12.93$; $p < 0.05$).

Effects of RAGE 11E6 on pain hypersensitivity in the TNI model of neuropathic pain

To determine the degree to which neutralization of RAGE contributes to chronic pain hypersensitivity induced by nerve injury, we performed nociceptive behavioral tests in the presence of a RAGE neutralizing antibody. TNI significantly reduced the pressure

and tactile withdrawal thresholds of the hind paw on the ipsilateral side (**Fig. 5**; $n=3$, 70.54 ± 1.37 mN; $p>0.05$) and a single systemic injection of RAGE 11E6 (10 mg/kg) had no effect on uninjured rodents (data not shown). Systemic administration of RAGE 11E6 failed to affect tactile hyperalgesia when administered at 7, 14, or 21 days. However, tactile withdrawal thresholds of the hind paw ipsilateral to TNI at PID28 were transiently returned to baseline levels by 4 hours (**Fig. 5**; $n=6-8$, 58.33 ± 2.21 mN, ANOVA; $p<0.01$). The finding that RAGE 11E6 did not elevate tactile hypersensitive levels above the pre-injury state shows that the animals ability to detect noxious tactile stimulus was not altered by the use of the antibody. Similar observations of RAGE 11E6 behavioral reversal in age-matched male rodents subjected to TNI was also observed (data not shown; $n=6-8$ per post injury time point).

Discussion

Our study provides evidence that the ligand at-HMGB1 acting through RAGE is a critical regulator of nociceptive signaling and sensitization *in vitro*. Moreover, as ongoing pain following peripheral nerve injury is thought to be maintained in part by activity in sensitized primary afferent neurons and could be influenced by an injury-induced upregulation of *de novo* receptors (White et al., 2005), latent upregulation of RAGE in the DRG corresponds with the ability of a monoclonal antibody against RAGE to modulate neuropathic pain-related behavior in the rat.

HMGB1 receptor signaling in the nervous system

There is growing evidence that the presence of cytosolic HMGB1 in the nervous system following insult contributes to neuropathologic neuronal excitability, including epilepsy, neuropathic pain and migraine (Maroso et al., 2010, Feldman et al., 2012, Karatas et al., 2013). A number of pain studies have demonstrated that the perisciatic or intrathecal administration of HMGB1 can produce rapid and transient mechanical and thermal hyperalgesia in rodents (Chacur et al., 2001, O'Connor et al., 2003, Shibasaki et al., 2010). However, given the possibility that either of the two isoforms of HMGB1 may be present after injury to the nervous system (Zandarashvili et al., 2013), there is little knowledge regarding which of the cognate HMGB1 receptors, RAGE and TLRs, contribute to changes in neuropathological conditions. Although sensory neurons exhibit both functional TLR4 and RAGE receptors, our studies show that RAGE 11E6 completely inhibits at-HMGB1-dependent neuronal excitability *in vitro*. In contrast, there is no evidence to

suggest that at-HMGB1 activates neuronal TLR4, considering a small molecule inhibitor of TLR4 failed to reverse at- HMGB1 elicited neuronal excitability *in vitro*.

RAGE signaling in the nervous system

HMGB1 acting through RAGE was initially implicated in neurite outgrowth in embryonic neurons and migration of numerous immune and non-immune cells types during states of inflammation (Rauvala and Pihlaskari, 1987, Merenmies et al., 1991, Fages et al., 2000, Rouhiainen et al., 2004). The relevance of HMGB1/RAGE to inflammation is relatively well described for the pathogenesis of diabetes, cardiovascular disease and various cancers (Rouhiainen et al., 2013). Whether HMGB1 activation of neuronal RAGE and the resultant increase in neuronal excitability during or following nerve injury-induced inflammation, represents a neuropathological consequence, is generally unknown. Initial reports in mice suggest that pharmacological and genetic blockade of RAGE impairs both innate immune response and nerve regeneration following sciatic nerve crush (Rong et al., 2004a, Rong et al., 2004b). Though the degree to which this injury altered tactile hyperalgesic behavior was not tested, the potential effects may not have manifested measurable changes given that both the nerve conduction velocities and gait analysis were tested for only three weeks post-nerve injury while the behavioral effects of increased RAGE expression in the rat DRG following peripheral nerve injury could only be adequately assayed at four weeks. A mechanism that might account for this latent change in RAGE could be due to a HMGB1 feed forward mechanism that exists in some immune cells and could serve to increase the levels of RAGE ligands and subsequently increased RAGE expression (Akirav et al., 2012).

Oxidation State dependent HMGB1 signaling in the nervous system

Functional delays in the expression of RAGE in the affected DRG and the latent ability of RAGE neutralization to reverse tactile behavior in the rodent may also be dependent on changes in the state of HMGB1. It is well known that HMGB1 may form a complex with other molecules to enhance proinflammatory responses, including LPS, IL-1, bacterial DNA, CXCL12, and viral RNA (Sha et al., 2008, Campana et al., 2009, Hreggvidsdottir et al., 2012). The state-dependent characteristics that contribute to protein/receptor interaction appear to depend on the molecular properties of three cysteine residues; the redox state of cysteine (C) 106, and a disulfide bond between C23 and C45 (Hoppe et al., 2006, Sahu et al., 2008). For example, during active inflammation the predominant form of HMGB1 is C106 thiol (all-thiol) and disulfide bond C23-45 (disulfide-HMGB1); however, when inflammation begins to subside, HMGB1 terminally oxidizes its cysteine residues and greatly diminishes biological activity (oxidized-HMGB1) (Antoine et al., 2010, Vezzoli et al., 2010, Yang et al., 2011). Moreover, an oxidizing environment following inflammation or injury may promote HMGB1 cytokine activity (Vezzoli et al., 2011). These changes in HMGB1 conformation over time combined with the feed forward expression mechanisms that exist may account for the delayed influence observed in this neuropathic pain model.

Several published works have shown the importance of HMGB1 in the propagation of pain pathology, and when combined with the duality of function of the HMGB1 molecule, the change of the expression of the RAGE receptor as time progresses *in vivo*, as well as the efficacy of the antibody against the RAGE receptor with pain

reversal, the conclusion can be drawn that RAGE and its signaling pathway are integral to pain pathology at later stages. Results of further study regarding the RAGE signaling axis may uncover targets for novel therapeutics and possibly have direct impact upon current clinical practice, through the discovery of potential biomarkers for pathologies of chronic inflammation, and through better understanding of the mechanisms of inflammation as they relate to disease. RAGE could possibly be a lynchpin to understanding the complete pathway leading to the pathogenesis of neuropathic pain.

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Figure Legend

Figure 2-1. RAGE, TLR4, IB4 and CGRP immunohistochemistry in primary sensory neuron cultures. Images depicting corresponding images of the cell nucleus label DAPI (**A**), RAGE (**B**) and TLR4 (**C**). Note that the majority of RAGE immunoreactive neurons are also positive for TLR4 (yellow arrows indicates co-expression). Corresponding images of DAPI (**D**), RAGE (**E**), and the non-peptidergic marker of nociceptive neurons, IB4 (**F**). A minority of IB4 cells exhibit RAGE (yellow arrow indicates co-expression). Corresponding images of DAPI (**G**), RAGE (**H**), and the peptidergic marker of nociceptive neurons, CGRP (**I**). Note that there are numerous RAGE immunoreactive neurons which are also positive for CGRP. Scale bar is 50 μ m.

Figure 2-2. RAGE neutralizing antibody, but not the TLR4 small molecule inhibitor, Compound 15, suppresses at-HMGB1-dependent neural excitation in acutely dissociated sensory neurons. Current clamp recordings were performed on small-to-medium (>30 μ m - >40 μ m) diameter lumbar 4–5 DRG neurons from naive rats. Firing of 1–2 action potentials (Tesana et al.) was elicited by a 1 second depolarizing current injection (ranging from 0.1 to 2.0 nA depending on the cell) every 30 seconds. Representative recordings demonstrating that application of at-HMGB1 (27 μ M) increases the number of elicited action potentials in DRG sensory neurons is not reversed by TLR4 small molecule inhibitor (compound 15) (**A**) or a TLR2 inhibitor (CU-CPT22) (**E**). In contrast, exposure to RAGE antibody effectively suppresses at-HMGB1-dependent action potential (**C**). Group data showing that compound 15 (**B**) and CU-CPT22 (**F**) do not reverse at-HMGB1-elicited increase in DRG neuron action potential firing while RAGE Ab does

reverse increased excitation (**D**). Representative recording demonstrating that application of LPS (1 µg/ml) increases the number of elicited action potentials in DRG sensory neurons which is unaffected by RAGE antibody (**G**). In contrast, the neuronal effects of LPS can be suppressed with compound 15 (**I**). Group data showing that compound 15 (**J**), but not RAGE Ab (**H**), reverses LPS-elicited increase in DRG neuron action potential firing. (Ligand treatment compared to receptor inhibitor; *p < 0.05)

Figure 2-3. Tibial Nerve Injury (TNI) alters the expression of neuronal transcripts in dorsal root ganglion (DRG) derived from TNI rats. (A – D) RT-PCR analysis showing the mRNA expression profile of CaV alpha2delta1 (**A**) Nav1.8 (**B**) and RAGE (*p< 0.05) (**C**) at different time points following TNI; post injury day (Holmbeck et al.) 14 and PID 28 (n=3). RT-PCR data were analyzed using the Ct method and mRNA expression levels are expressed relative to L27- ribosomal housekeeping gene.

Figure 2-4. RAGE protein expression following TNI at post injury day 28. Immunoblot of RAGE in L4/5 DRGs from naïve, sham injured and TNI ipsilateral to the injury at [PID] 28 (n=3, sham versus injury; *p< 0.05). Actin was used as a loading control to which samples were normalized.

Figure 2-5. Decreased tactile hyperalgesia following intraperitoneal injection of a neutralizing RAGE antibody in tibial nerve-injured rats at Day 28. Paw withdrawal threshold (PWT) in rodents subjected to TNI (n = 6-8, white bars) were significantly reduced when compared with pre-TNI thresholds (n = 6-8, black bar) for at least 28 days. Administration of control, non-neutralizing antibody did not alter PWT at baseline or after

TNI at 7, 14, 21 or 28 days (n = 6-8, CT Ab; gray bars). A humanized monoclonal antibody to RAGE (RAGE Ab; 10 mg/kg body weight; n = 6-8 each) was administered intraperitoneally and ipsilateral PWT was assessed using the von Frey filament test. Behavior was tested at 1 h post injection and again at 4 hours (data not shown). A single injection of RAGE Ab did not produce a change in PWT at TNI PID 7, 14, and 21 (striped bar) that differed from pre-RAGE Ab (white bars). At TNI PID 28 a single injection of RAGE Ab successfully reversed TNI decreases in PWT when compared with pre-RAGE Ab treatment (*p< 0.05).

Figure 2-1

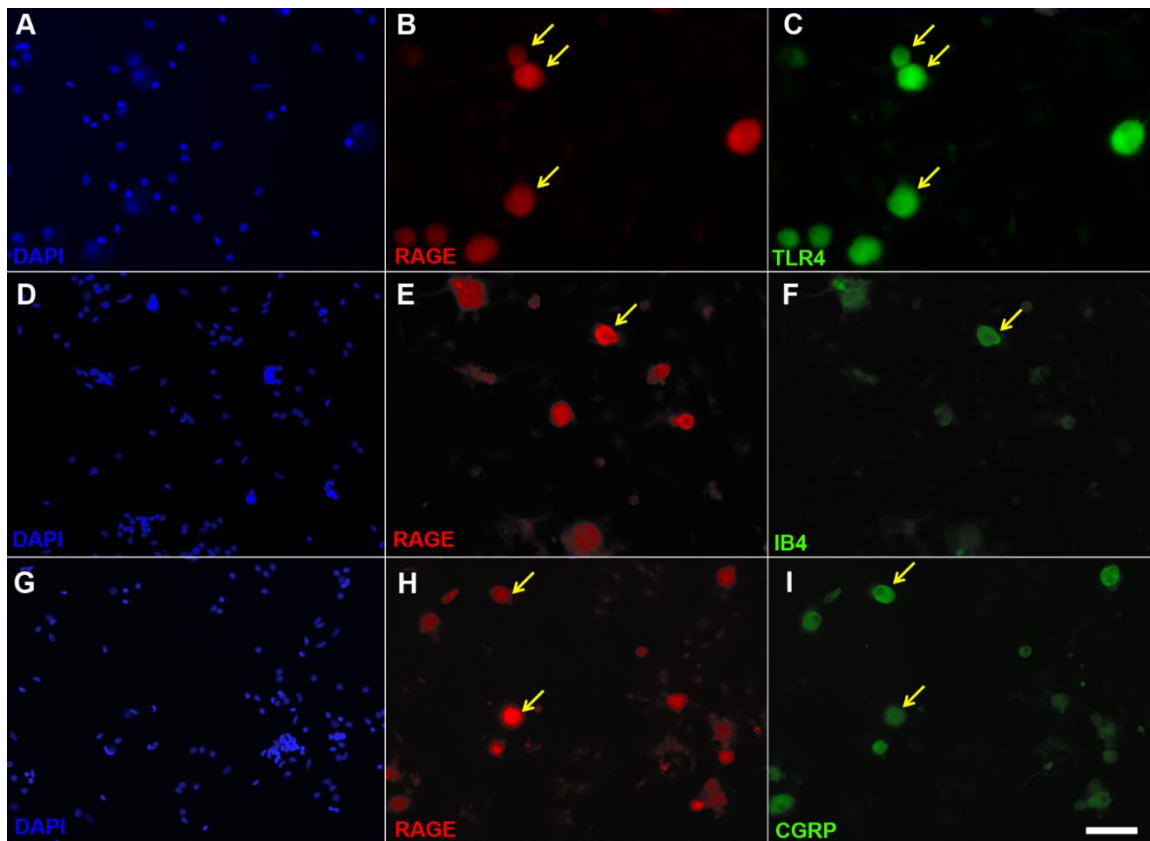


Figure 2-2

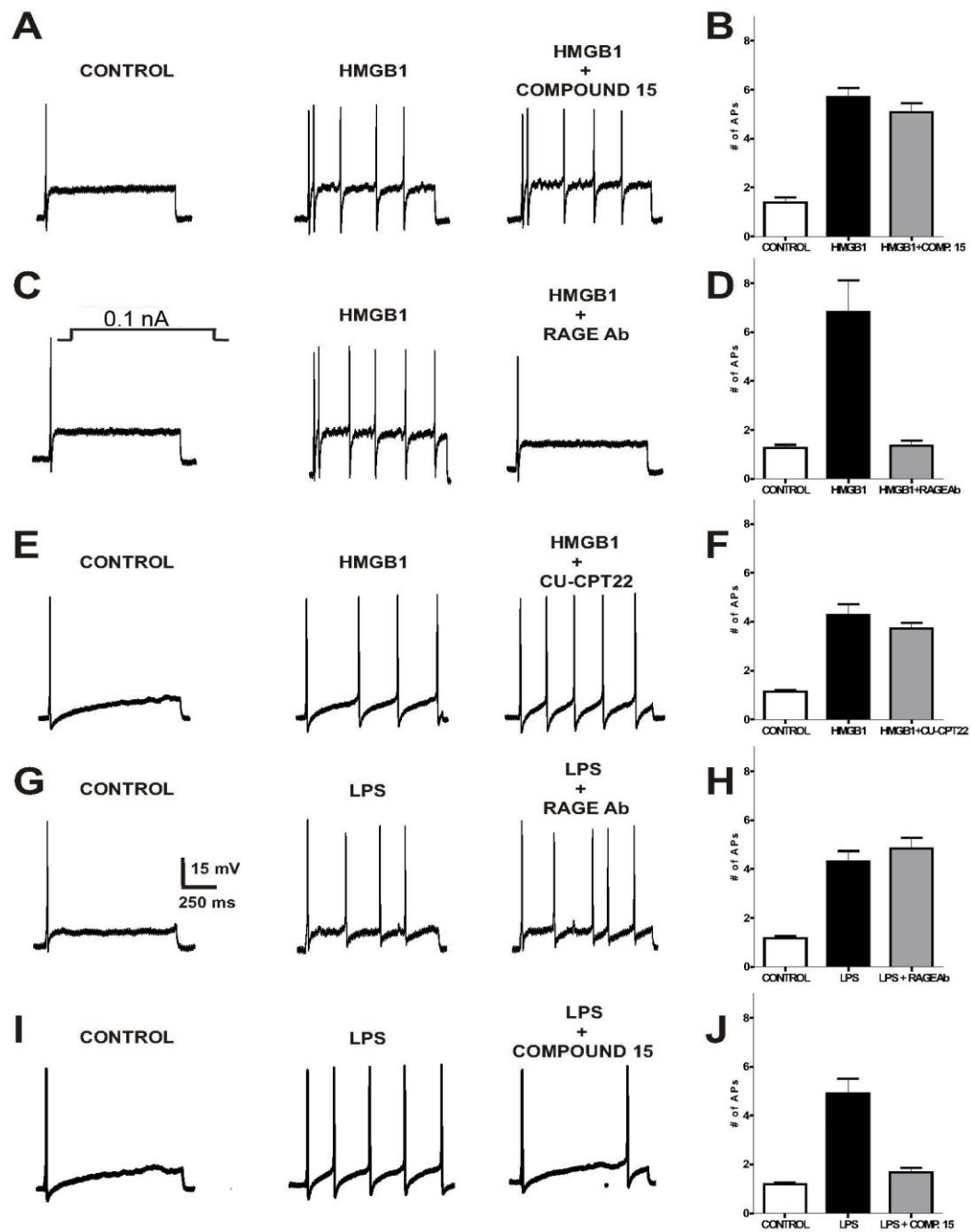


Table 1

Differential increases in neuronal $[Ca^{2+}]_i$ by oxidation specific HMGB1

	Percent Total Neurons	Percent Responsive to LPS
at-HMGB1	14.58% (14/96)	43% (6/14)
ds-HMGB1	7.55% (8/106)	100% (8/8)

Table 2

Population of nociceptive neurons via capsaicin sensitivity responsive to HMGB1 isoforms

<i>N</i> = 217	Percent Responsive to capsaicin
at-HMGB1	70.1%
ds-HMGB1	42%

Figure 2-3

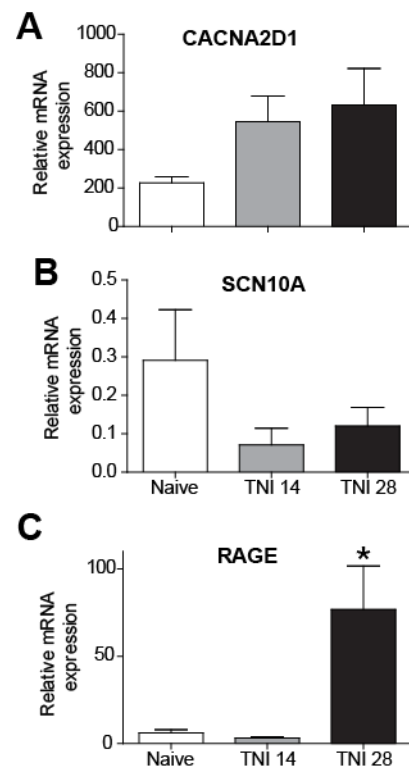


Figure 2-4

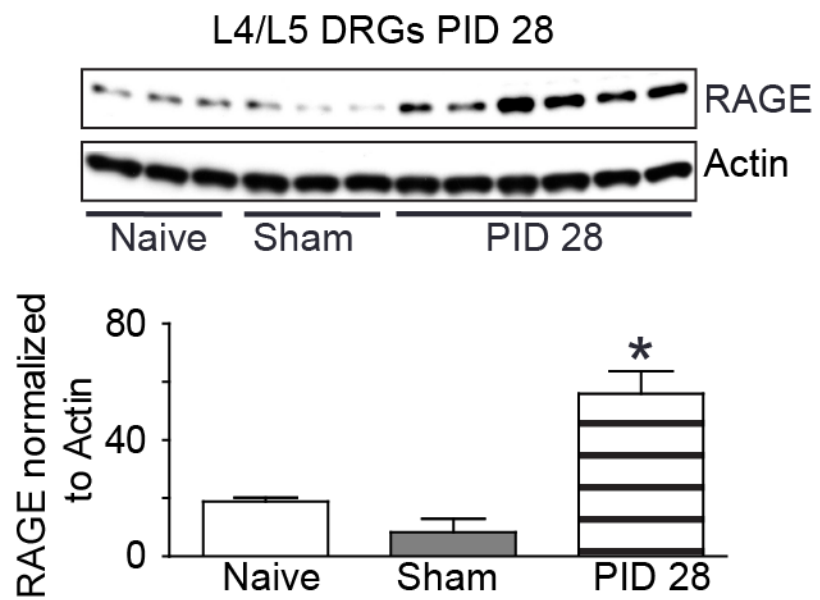
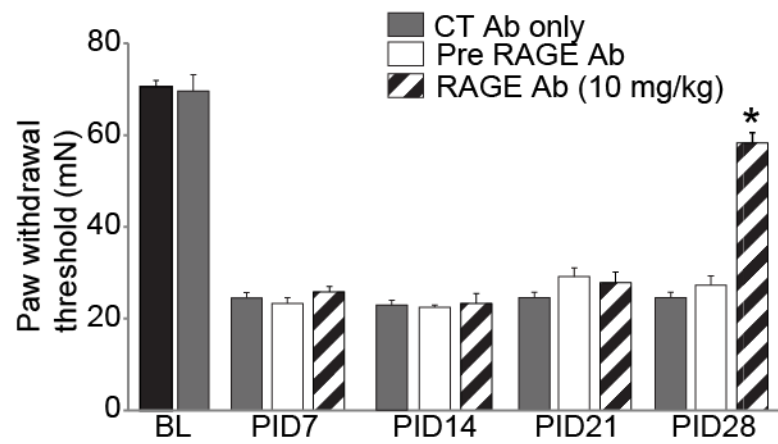


Figure 2-5



Chapter 3: Neuronal Excitation and the TIR Adaptor Proteins

Nociception as a function of MyD88-dependent signaling in a model of Opioid-Induced Hyperalgesia

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Material in this chapter currently under preparation for journal submission.

Introduction

Opioid analgesics are primarily known for treating severe pain, but these beneficial effects are often accompanied by sedation, respiratory depression, constipation and the paradoxical phenomenon known as opioid-induced hyperalgesia (OIH). Though clinically used opioids are known to bind to GPCR opioid receptors and produce analgesia, this class of drugs also creates a neuroinflammatory response which can compromise pain relief (Hutchinson et al., 2011).

Investigations of opioid-induced neuroinflammation suggest that both the natural alkaloids found in the resin of the *Papaver somniferum* and synthetic substances can activate a non-GPCR known as toll-like receptor 4 (TLR4). Early work by Roy and colleagues first established that low dose morphine in combination with LPS potentiated activation of NF- κ B and pro-inflammatory cytokine production. Conversely, naloxone could block the morphine augmentation of cytokine production by LPS stimulated macrophages (Roy et al., 1998). More recent studies demonstrate that morphine and its metabolite, morphine-3-glucuronide (M3G), binds to TLR4 and its accessory protein, myeloid differentiation protein 2 (MD-2), triggering signaling cascades which lead to pro-inflammatory cytokine production in macrophages and microglial cells (Lewis et al., 2010, Wang et al., 2012).

TLR4-mediated effects by LPS and opioids/metabolites are not restricted to immune cells as small nociceptive neurons bearing TLR4 can elicit pro inflammatory cytokine and prostaglandin production, modulation of opioid-related peptides and activity dependent release of neuropeptides (Hou and Wang, 2001, Acosta and Davies,

2008, Tse et al., 2014). More importantly, these same TLR4 agonists produce rapid increases in neuronal calcium flux, elevated states of excitability and potentiation of voltage-gated sodium current in nociceptive sensory neurons (Wadachi and Hargreaves, 2006, Ochoa-Cortes et al., 2010, Diogenes et al., 2011b, Due et al., 2012b, Due et al., 2014). Taken together, the deleterious properties of opioid-induced TLR4 activation in neurons and glial cells can produce stimulus-dependent behavioral tactile and thermal hyperalgesia (Lewis et al., 2010, Due et al., 2012b, Eidson and Murphy, 2013, Due et al., 2014).

TLR4 is comprised of an extracellular domain with multiple leucine-rich repeats, a single transmembrane helix, and an intracellular region approximately 150 amino acids in size and comprised largely of a Toll/IL-1R (TIR) resistance domain (Medzhitov et al., 1997, Rock et al., 1998). Interactions of TIR domains with TLRs such as TLR4 are pivotal in the early stages of inflammatory signaling including inhibition of NF- κ B translocation and early IL-1 β mRNA expression in LPS-stimulated macrophages (Toshchakov et al., 2007). Four TIR-containing adapter proteins including MyD88, TIR domain-containing adapter protein [TIRAP], TIR domain-containing adaptor inducing IFN- γ [TRIF]–related adaptor molecule [TRAM], and TRIF are responsible for propagation of signal to downstream targets (Horng et al., 2001, Horng and Medzhitov, 2001, Kagan et al., 2008). Signal transduction of the assembled TLR signaling complex is achieved through the coordination of the initial TIR domain interactions that mediate adapter recruitment to the receptor TIR (Kagan and Medzhitov, 2006).

To diminish TLR4 agonist-dependent events *in vitro* and nociceptive behavioral changes due to opioids *in vivo*, we assayed the ability of a select group of cell permeating decoy peptides to affect LPS-induced neuronal activation and M3G-dependent hyperalgesia in rodents. The protein-protein interface targeted by the decoy peptides used a conserved structural feature, the BB loop, and functionally inhibited LPS signaling in primary murine macrophages (Toshchakov et al., 2007, Toshchakov et al., 2011). The data presented in this study identify decoy peptides that target TLR4 TIR and provide evidence for the mechanism of decoy peptide action in both dissociated nociceptive sensory neurons and rodent OIH.

Methods

Animals

Pathogen-free, adult female and male Sprague-Dawley (S/D) rats (150–200 g; Harlan Laboratories) were housed in temperature ($23 \pm 3^{\circ}\text{C}$) and light (12-hr light: 12-hr dark cycle; lights on at 07:00AM) controlled rooms with standard rodent chow and autoclaved tap water available. Experiments were performed during the light cycle. Animals were randomly assigned to the treatment groups. All animal related experiments were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines established by the International Association for the Study of Pain.

Reagents

All reagents were freshly prepared in buffer the day of experimentation. Capsaicin and lipopolysaccharide (biologically reactive serotype B5:55) were obtained from Sigma and reconstituted in deionized H_2O (Sigma; St. Louis, MO). The culture media used for acute dissociation cultures was made of Ham's F-12 mixture, DMEM, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 $\mu\text{g}/\text{ml}$ and 100U/ml), and N2 (Life Technologies, Corp.). Decoy peptide sequences for 4BB, 4aE, 4R1, 4R3, and 4R9 (Toshchakov et al., 2011) were modified to include a TAT (transduction domain of human immunodeficiency virus-1 (HIV)) segment in order to aid in membrane permeability in our neuronal cell cultures (Schwarze et al., 1999, Brittain et al., 2011). Morphine-3- β -D-

glucuronide (M3G) was supplied by NIH/NIDA Drug Supply Program and utilized at a concentration (10mg/kg, i.p.) (Due et al., 2012b).

Preparation of acutely dissociated dorsal root ganglion neuron

In order to maintain a nociceptive population in the primary cell culture, the L4, L5, and L6 dorsal root ganglia (DRG) were removed via dissection post mortem following the methods outlined by Ma and Lamotte (Ma and LaMotte, 2005). The DRGs were dissected and placed in a test tube containing DMEM and neutral protease and collagenase (Worthington Biochemical), while gently rocking at 37°C for 40 minutes. The cells were then dissociated by mechanical trituration, in culture media containing 1 mg/ml bovine serum albumin and trypsin inhibitor (Worthington Biochemical). The cells were then plated on coverslips coated with poly-L lysine and laminin (BD Biosciences) and incubated for two to three hours before more culture media was added to the wells. The cells were then allowed to sit undisturbed for 12 to 15 hours to adhere at 37°C (with 5% CO₂).

Ca²⁺ imaging

Dissociated DRG cells were loaded with fura-2 AM (3mM, Invitrogen Corp.) for 25 min at room temperature in a balanced sterile salt solution (BSS) (NaCl 140mM, Hepes 10mM, CaCl₂ 2mM, MgCl₂ 1mM, glucose 10mM, KCl 5mM). The cells were rinsed with the BSS and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera coupled to a microscope and MetaFluor software (Molecular Devices Corp.).

Cells were illuminated with a 150 W xenon arc lamp, and the excitation wavelengths of the fura-2 (340/380nm) were selected by a filter changer. Sterile solution was applied to cells prior to treatment, any cells that responded to buffer alone were not used in neuronal responsive counts. The cells were assayed for the ability of the decoy peptide to interrupt/reverse the ability of LPS to elicit a calcium flux from the cell. The treatments were given in the order of LPS (1-2 μ g/mL), the decoy peptide (15 μ M), a second treatment of LPS (1-2 μ g/mL), and capsaicin (3nM). A minimum period of three minutes was allowed for observation after each treatment was introduced to the bath; the next treatment would only be administered if the signal re-established a baseline signal output. Calcium imaging traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts.

Electrophysiology

Sharp electrode intracellular recordings were obtained from primary afferent neurons 12 to 18 hours after acute dissociation. Coverslips were transferred to a recording chamber mounted on the stage of an inverted microscope (Nikon Eclipse Ti; Nikon Instruments, Inc.). The chamber was perfused with a bath solution (NaCl 120mM, KCl 3mM, CaCl₂ 1mM, MgCl₂ 1mM, Hepes 10mM, Glucose 10mM) adjusted to a pH 7.4 and osmolarity of 300 Osm. All recordings were obtained at room temperature. Intracellular recording electrodes were fabricated from borosilicate glass (World Precision Instruments: Sarasota, FL) and pulled on a Flaming/Brown micropipette puller (P-98, Sutter Instruments). Electrodes were filled with 1.0M KCl (impedance: 40–80 M Ω) and

positioned by a micromanipulator (Newport Corp.: Irvine, CA). A current injection of -0.1nA was used to bridge-balance the electrode resistance.

Prior to electrode impalement, the size of the soma to be recorded was classified according to its diameter as small ($\leq 30\mu\text{m}$), medium (31 to $45\mu\text{m}$) and large ($\geq 45\mu\text{m}$). Electrophysiological recordings were performed with continuous current-clamp in bridge mode using an AxoClamp-2B amplifier, stored digitally via Digidata 1322A interface, and analyzed offline with pClamp 9 software (Axon Instruments, Inc.). A neuron was accepted for study only when it exhibited a resting membrane potential (Balermipas et al.) more negative than -45mV . For each neuron isolated for study, a continuous recording was obtained for one minute without the delivery of any external stimulus. Neuronal excitability of small and medium diameter dissociated DRG sensory neurons was measured by injecting one second current pulses into the soma every 30 seconds. Current was adjusted in order to elicit one to two action potentials per current injection under baseline conditions. Following 3 control current injections, LPS ($1\text{-}2\mu\text{g/mL}$) was applied to the coverslip and current injections continued every 30 seconds; coverslip(s) may have been exposed to decoy peptide ($15\mu\text{M}$) prior to LPS administration depending on the experiment. Neuronal excitability was measured as number of action potentials elicited per current pulse before and after addition of LPS. If LPS increased neuronal excitability, decoy peptides or vehicle was added to the bath to determine if LPS-elicited neuronal excitation could be reversed.

Behavioral assessment

All rodents were habituated to testing chambers for at least two days. Rodents were randomly assigned to sham or injured test groups. All baseline testing occurred before and after exposure to reagents. The incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hindpaw was measured with custom-designed Von Frey filaments capable of exerting forces of 10, 20, 40, 60, 80 and 120mN and a uniform tip diameter to designated loci present on the plantar surface of the foot (Bhangoo et al., 2007). The filaments were applied six times in order of ascending force to each hind foot of the rodent. The von Frey withdrawal threshold was defined as the force that evoked a minimum detectable withdrawal observed on 50% of the tests given at the same force level. All behavioral assays were performed by double-blinded individuals.

Statistics

Data are presented as group mean \pm SEM. Tactile threshold time course curves (plotted as the mean \pm SEM vs. time after treatment) were analyzed with a one-way analysis of variance (ANOVA) with repeated measures over time, followed by Dunnett's post hoc test to compare each time point to the same group's baseline. All analyses employed Prism statistical software, CA, USA.

Results

Recent studies targeting surface-exposed segments of TLR4 TIR domain protein with decoy peptides effectively inhibit receptor-mediated signaling (Toshchakov et al., 2011). To test the degree to which TLR4 TIR domain diminish excitatory events in LPS responsive sensory neurons we utilized five decoy peptides, 4R9, 4R3, 4aE, 4R1 and 4BB which are known to potently inhibit TLR4-mediated events in LPS-stimulated macrophages (Toshchakov et al., 2011). All of these decoy peptides exhibited approximately equal length and a surface-exposed segment of TLR4 TIR primary sequence. The peptide sequences were modified to include a TAT precursor at the N-terminus of each peptide, in order to assist bypassing the neuronal cell membrane (Brittain et al., 2011).

TIR-binding decoy peptides decreases LPS induced Ca^{2+} mobilization in dissociated sensory neurons

Activation of TLR4 expressed by primary sensory neurons results in the increase in the intracellular Ca^{2+} concentration (Due et al., 2012b). In the first series of experiments, assessed neurons were selected for morphological qualities (small ($\leq 30\mu m$) and medium (31-45 μm) diameter cells) and exposed to LPS in the absence or presence of the five decoy peptides after which capsaicin (3 nM) was added to characterize the responsive neurons. A positive response to capsaicin indicates the cell is a nociceptor expressing the TRPV1 channel (O'Neill et al., 2012).

We observed that LPS application increased $[Ca^{2+}]_i$ in a moderate number of DRG sensory neurons (27%; n=575 cells) derived from female rodents (n=8). Application of the decoy peptides interrupted TLR4 neuronal activation to different degrees. The decoy peptide 4BB yielded inhibition in 71% of LPS-sensitive neurons; the decoy peptide 4aE elicited a 56% rate of inhibition, and the 4R9 peptide inhibited 48% of LPS-sensitive cells. Peptide 4R1 and 4R3 demonstrated a blockade of LPS-sensitive signal in 41% and 18% of cells, respectively (**Fig 1** and **Table 1**).

Numerous LPS-responsive cells, which were sensitive to the decoy peptide exposure, did not respond to capsaicin. Only 33% of LPS-sensitized neurons were affected by 4R9 followed by 4BB (24%) and 4R3 (18%). A sub-population of LPS-responsive neurons TRPV1 cells exhibited presence of the decoy peptides. 4aE and 4R1 peptides were limited to only a 17% and 12% block of LPS- and capsaicin-sensitive neurons, respectively (**see Table 1**).

TIR-binding decoy peptides diminish inward current facilitation in nociceptive neurons

TLR4 agonists such as LPS have been shown to elicit inward current in nociceptive sensory neurons and increase the excitability of sensory neurons (Ochoa-Cortes et al., 2010, Diogenes et al., 2011b, Due et al., 2012b) and activation of TLR4-mediated excitation in nociceptive neurons increases density in a number of voltage-gated sodium currents (Due et al., 2012b). To determine the degree to which the decoy peptide 4BB inhibited LPS-induced neuronal excitation, we examined neuronal response using sharp electrodes in current clamp mode.

Repeated current pulse combined with LPS administration produced a significant increase in the excitability of small diameter neurons when compared to baseline levels, though less than 27% of neurons responded to LPS (n=49). We also observed 1.6 ± 0.2 APs in cells under control conditions compared to 4.8 ± 0.6 APs in cells subjected to LPS (n = 13) (**Fig. 2A**). Subsequent treatment with decoy peptide 4BB completely blocked LPS-dependent excitability in all sensory neurons that responded to LPS (1.2 ± 0.2 APs for 4BB, n = 13; F = 41.82, $p < 0.05$; Dunnett's multiple comparison test, $P < 0.05$) (**Fig. 2B**).

Pretreatment of rodents with decoy peptides prevents the rapid induction of tactile hyperalgesia due to systemic M3G administration

It has been noted in rodents that the level of antinociception achievable with morphine or morphine analogues is reduced due to glial and neuronal TLR4 activation (Lewis et al., 2010, Due et al., 2012b, Due et al., 2014). To further explore the role of TLR4 signaling in rodent OIH, we examined the degree to which rats treated with a combination of M3G and decoy peptides produced changes in tactile behavioral assays. M3G administration (10 mg/kg, i.p.) produced significant reductions in the paw withdrawal threshold to tactile stimulus (**Figure 3-3A-E**; $P < 0.05$). In contrast, paw withdrawal threshold to tactile stimulus in rodents pretreated with either 1 or 10 mg/kg, i.p. of 4BB or 4aE 30 minutes prior to M3G administration did not differ from baseline thresholds or the combination of decoy peptides plus vehicle (**Figure 3-3A,B**; n = 12, F = 47.62, $P < 0.05$; Dunnett's multiple comparison test, $P < 0.05$). The decoy peptide effect in M3G-treated animals was not apparent 4 hours after treatment for either 4BB or 4aE (data not shown).

Decoy peptides 4R1, 4R3 and 4R9 failed to produce changes in paw withdrawal threshold in M3G-treated animals at either dosage (**Figure 3-3C-D**).

Discussion

This study explored the functional basis of nociceptive neuronal TLR4 signaling associated with the bacterial cell product, LPS and the morphine metabolite, M3G. Activation of TLR4 present on nociceptive neurons produces rapid changes in intracellular calcium, action potential discharge, potentiation of voltage-gated sodium currents and activity dependent release of neuropeptides (Hou and Wang, 2001, Due et al., 2012b). More importantly, the known TLR4 agonist, M3G, elicits increased neuronal excitation and behavioral hyperalgesia (Due et al., 2012b). Though several mechanisms may be involved with the neuronal TLR4-mediated events, we demonstrate for the first time that interruption of the TIR-dependent recruitment of the cytoplasmic adaptor protein MyD88 using the decoy peptide 4BB is instrumental for eliminating both M3G-mediated excitation in disassociated nociceptive neurons and behavioral hyperalgesia. Similar observations were apparent for the decoy peptide, 4aE.

MyD88 is a necessary signaling adapter protein for all members of the IL-1R family and all TLRs with the exception of TLR3 (Andreaskos et al., 2004, Nagpal et al., 2009). Previous observations found that when cell-permeating decoy peptides bound to various surface-exposed segments of the MyD88 both early cytokine mRNA expression and MAPK activation could be inhibited in LPS-stimulated murine macrophages (Toshchakov et al., 2011). Similar evidence using TIR mimetics was observed in IL-1 β stimulated anterior hypothalamic neurons (Davis et al., 2006). Herein, we observed that co-exposure of the five decoy peptides known to inhibit protein function in stimulated macrophages diminished LPS-mediated calcium fluxes in a rank order of 4BB > 4aE > 4R9 > 4R1 > 4R3.

In particular, the degree to which the decoy peptide 4BB affected both neuronal excitation and opioid metabolite-induced behavioral hyperalgesia appears to provide strong evidence that neuronal TLR4 TIR dimerization is extremely important for excitation in nociceptive sensory neurons.

Induction of the TLR4 signaling cascade occurs via two pathways which both contribute to the synthesis and release of pro-inflammatory cytokines. Immune cell MyD88-dependent cascades tends to activate NF- κ B in the early stages of injury or infection while MyD88-independent signaling is dependent on TIR domain-containing adaptor-inducing IFN- β (TRIF) and leads to NF- κ B activation in later stages (Yamamoto et al., 2002, Mansell et al., 2004). Transcriptional changes due to LPS or M3G results in the induction of cytokines in the CNS through receptor-mediated assembly of the signaling adapter proteins, TIR and MyD88 (Lewis et al., 2010, Grace et al., 2014). Though the cytokines associated with TLR4 activation in DRG neurons also elicit an increase in TNF α , and COX2 through activation of NF- κ B there is no evidence to suggest that these actions are TRIF dependent (Tse et al., 2014).

Although peripheral nociceptor activation by TLR4-mediated mechanisms likely underlies behavioral sensitivity to noxious stimuli following nerve injury or drug-induced changes, little is known regarding the mechanism. Our data support a role for MyD88 in regulating the off-target behavioral effects of morphine. However, the balance of nociceptor activation by nerve injury or inflammation may take other forms of the TLR4-associated signaling adapter proteins. For example, MyD88/TRIF signaling-deficient or MyD88-deficient mice fail to exhibit tactile allodynia following spinal nerve ligation

(Stokes et al., 2013). Similar loss of allodynia is present in genetically modified rodents subjected to intrathecal LPS or drugs associated with chemotherapy-induced peripheral neuropathy (Stokes et al., 2011, Park et al., 2013, Li et al., 2014, Woller et al., 2015) including mice deficient for a signaling adapter downstream to both TLR2 and TLR4, TIR domain-containing adaptor protein (TIRAP; also known as Mal) (Stokes et al., 2013).

The ability of LPS administration to elicit intracellular calcium mobilization in both capsaicin- and non-capsaicin-sensitive neurons predicts that these sensitized cells may convey thermal or mechanical modalities independent of inflammation states (Drew et al., 2002). Though not distinguished herein, these LPS sensitive cell subpopulations are divided into peptidergic, TRPV1-positive and non-peptidergic, IB4-positive neurons (Due et al., 2012). Moreover, transactivation of the TRPV1 channel through TLR4 stimulation has been shown a potential mechanism of nociceptive response (Diogenes et al., 2011b) and these TRPV1-bearing cells may not be only capable of sensing thermal changes as a number of these neurons coexpress TRPA1 (Story et al., 2003, Vilceanu and Stucky, 2010), but also may be cross-sensitized by the presence of TRPA1 agonists (Spahn et al., 2014). Interestingly, the presence of supraphysiological concentrations of LPS activates TRPA1 sensitive neurons independent of TLR4 (Meseguer et al., 2014). Similar TLR-independent actions have also been described for bacterial N-formylated peptides and the pore-forming toxin α -haemolysin (Chiu et al., 2013). The degree to which the TLR4 agonist, M3G, preferentially activates capsaicin- and non-capsaicin-sensitive neurons is unknown.

Other ion channels which may be associated with TLR4 activation include voltage-gated sodium channels. It is known that the M3G elicits a substantial increase in the

current density for voltage-gated sodium channels (NaV), NaV1.6, NaV1.7 and NaV1.9, but not NaV1.8 (Due et al., 2012b), which can be pharmacologically inhibited by the state-dependent sodium channel blocker, carbamazepine (Due et al., 2014). Some of these same Na⁺ currents may also influence other TLR4-sensitive cell types such as macrophages and microglia (Black et al., 2009) and may also contribute systemic inflammatory conditions (Schaper et al., 2013). Together the pharmacological targeting of TLR4 signaling adapter proteins may provide insight into opioid-sparing therapies and neuropathic pain disorders.

Conclusion

Given the importance of the immune system, it is impractical to suggest that TLR4 receptor blockade can provide complete elimination of OIH. However, targets such as MyD88 and TIRAP could have a greater effect on the off-target effects of opioids and elicit opioid-sparing in the clinical population (Fekonja et al., 2012). This study also has a larger range of application when considering that TLR4 shares its adaptor protein complex with another receptor known for neuronal excitation and activity, and innate immune functionality, RAGE (Sakaguchi et al., 2011). RAGE shares both the TIR domain and TIRAP and MyD88 adaptor proteins with TLR4, which suggests that is in fact susceptible to the same methods of inhibition via decoy peptide administration (Ibrahim et al., 2013). In addition, pharmacological techniques allow for changes in concentrations and dosing, meaning that total inhibition should not be the goal (Bevan et al., 2010). Mitigation of the ability of TLR4 to recruit its adaptor proteins and initiate downstream signaling may prove more successful at preventing pathological development, while maintaining the immune functionality of the receptor.

Figure Legend

Figure 3-1: Representative calcium imaging trace of decoy peptide assessment. Acutely dissociated neurons were assayed for activity via calcium imaging (FURA-2AM) in the presence of TLR4 agonists (LPS), decoy peptides for the MyD88 adaptor protein, and capsaicin. Administration of decoy peptide alone did not result in neuronal activation. Cells were first assessed for LPS sensitivity followed by application of the decoy peptide. A second dose of LPS was given to observe the effects of the peptide, and capsaicin was used to further characterize the neuronal population.

Figure 3-2: Representative current clamp trace. Current clamp recordings were performed on small-to-medium ($>30\text{ }\mu\text{m}$ – $>40\text{ }\mu\text{m}$) diameter lumbar 4–5 DRG neurons from naive rats. Firing of 1–2 action potentials was elicited by a 1 second depolarizing current injection (ranging from 0.1 to 2.0 nA depending on the cell) every 30 seconds (Tesana et al., 2012). Representative recordings demonstrating that application of LPS and the subsequent increase in elicited action potentials in DRG sensory neurons can be reversed by administration of MyD88 decoy peptides.

Figure 3-3: Changes in opioid-induced hyperalgesia following intraperitoneal injection of MyD88 decoy peptide. Paw withdrawal threshold (PWT) of rodents demonstrated a significant decrease following the administration of M3G and subsequent induction of opioid-induced hyperalgesia. Administration of decoy peptides targeting the MyD88 adaptor protein proved efficacious to varying degrees in returning PWT to baseline levels when introduced intraperitoneally. Peptides were administered at doses of

1mg/kg and 10mg/kg. Administration of peptides alone at either dose failed to alter baseline baseline PWT. The peptides 4BB (**A**), 4aE (**B**), 4R1 (**C**), 4R9 (**D**), and 4R3 (**E**) were assayed.

Figure 3-1

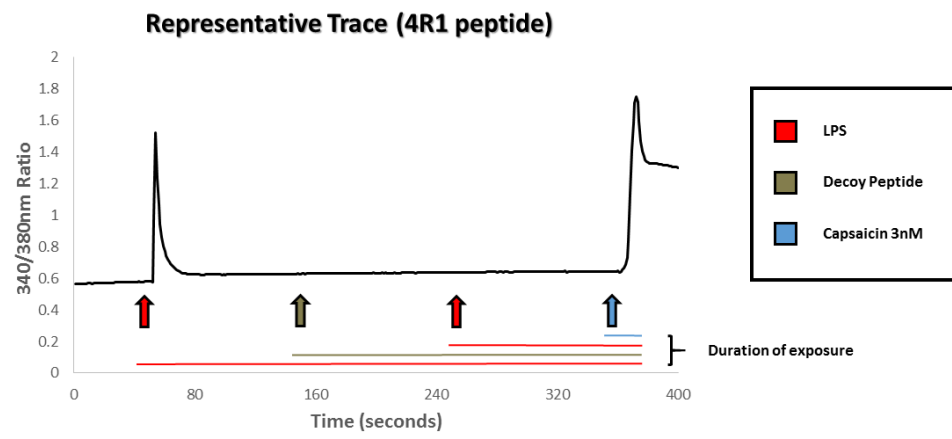


Figure 3-2

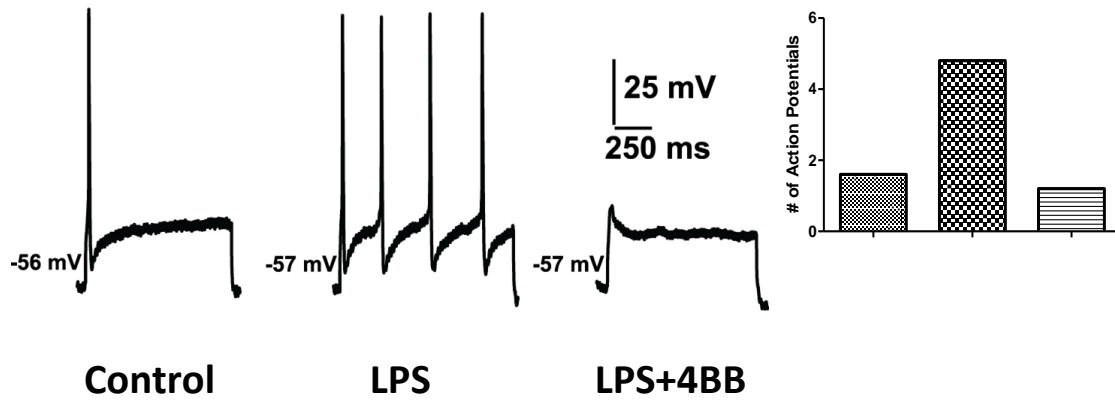


Figure 3-3

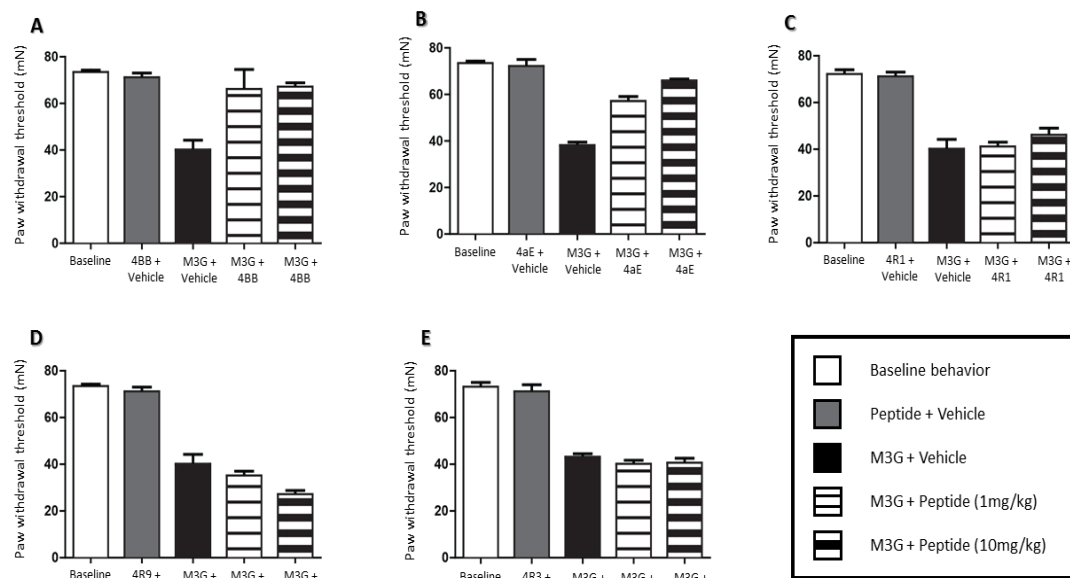


Table 1:

<u>Decoy Peptide</u>	<u>LPS-inhibition success rate</u> <i>(successful decoy inhibition to total LPS responding neurons)</i>	<u>Capsaicin sensitivity</u> <i>(TRPV1 activation to total number of decoy inhibited neurons)</i>
TAT-4BB (n = 99 cells)	27/38 – 71%	9/27 – 33%
TAT-4aE (n = 85 cells)	10/18 – 56%	3/10 – 30%
TAT-4R9 (n = 94 cells)	15/31 – 48%	10/15 – 67%
TAT-4R1 (n = 99 cells)	7/17 – 41%	2/7 – 29%
TAT-4R3 (n = 97 cells)	2/11 – 18%	2/2 – 100%

Differential mitigation of neuronal calcium flux as a result of decoy peptide administration and capsaicin-sensitivity.

Chapter 4: Project Discussion

Inflammation as a process is exceedingly complex, with multiple moving parts. Successful inflammation results in the resolution of disease or injury through coordinated activity between the injured tissue, immune cells, and the molecular ligands and receptors used for communication. However, inflammation can quickly develop into a maladaptive process as a result of imbalance in any of the aforementioned aspects. One such example can be seen with reactive oxygen species (ROS), which play a crucial role in the propagation of maladaptive inflammatory signaling. In the presence of microbial threats, ROS are important for the immune response (Azad et al., 2008). ROS also serve as the byproducts of cellular function, as seen with aerobic metabolism, and can also be produced through the activity of pro-inflammatory molecular signals (Brune et al., 2013). Low levels of ROS can in fact serve in a protective fashion to the sequelae of cellular aging (Kammeyer and Luiten, 2015).

Increases in ROS production can often contribute to pathological damage to DNA, RNA, and proteins (Chen and Nunez, 2010). This process contributes not only to cell dysfunction and necrosis, but in changing the oxidative nature of the extracellular space. As described earlier, HMGB1 is susceptible to reversible change due to extracellular conditions (Rubartelli and Lotze, 2007, Sahu et al., 2008, Venereau et al., 2012a). HMGB1 can experimentally be shifted from one state to another through the use of reducing and oxidizing agents (Hoppe et al., 2006). These conformational changes of HMGB1 have been observed to occur as a result of ROS activity, further propagating the development of chronic inflammation (Tang et al., 2012a). This ROS-induced conformation change can

lead to TLR4 signaling instead of RAGE, or inert HMGB1 and promotion of tolerance and inactivity from the affected tissue (Kazama et al., 2008, Lotfi et al., 2009).

If the extracellular environment can indeed have an effect upon HMGB1 conformation ability, this may suggest a method of localization for the DAMP and associated receptors as well. The immediate area of injury would be rife with inflammatory products and HMGB1 in both states (all-thiol and disulfide), leading to further release of these products and HMGB1. However, the time constraints associated with HMGB1's all-thiol form could possibly limit its effects in non-affected tissues (Zandarashvili et al., 2013). Combined with the fact that in the case of all-thiol HMGB1 and RAGE, splice variants of RAGE are present on several tissues, acting as 'heat sinks' and as another mechanism of signal control to prevent possible 'off-target' effects of HMGB1 (Jules et al., 2013). Soluble RAGE (sRAGE) is known to circulate the vascular system in certain conditions, and could act in the case of inflammation to limit HMGB1 effects outside of the insulted area (Mangalmurti et al., 2012).

Oxidation of HMGB1 would lead to a change in state to the TLR4-affiliated ligand disulfide HMGB1 and upon TLR4 binding, would result in the production of inflammation cytokines. The production of these inflammatory products would only serve to further aggravate the extracellular environment, as well as possibly continue the possible positive feedback relationship of ROS and disulfide HMGB1. Even in the absence of ROS, HMGB1 has been observed to evoke a positive feedback mechanism with not only further HMGB1 release, but the upregulation of the RAGE receptor as well (Sorci et al., 2012).

Furthermore, ROS can have a deleterious effect upon DNA, resulting in genetic damage and potentially facilitating HMGB1 binding to the newly-formed alternative DNA structure (Cooke et al., 2003). Still unknown about the aforementioned feed-forward mechanism of HMGB1 is the potential role the molecule plays in gene transcription. As HMGB1 binds to DNA, it not only can act as a transcription factor, it introduces a severe bend in the DNA segment by introducing a hydrophobic domain into the minor groove (Thomas and Travers, 2001). The additional distortion of the genetic structure beyond the HMGB1/DNA construct has been theorized to enhance gene transcription by allowing once distant transcription factors to interact in close proximity (Jayaraman et al., 1998). This change in transcriptional activity and protein production may have either a positive or negative impact upon inflammatory resolution. Regardless, considering that inflammation is a result of multiple moving parts and systems functioning in concert, it is well worth thorough investigation as a possible contributor to HMGB1-mediated pathology.

The effects of HMGB1 on neuronal signaling

Peptide inhibition of the MyD88 and TIRAP adaptor proteins proved efficacious for inhibition of both RAGE and TLR4 signaling as a result of HMGB1 binding in the sensory neuron. Not only does this provide a new target for mitigation of neuronal hyperactivity, it describes a level of importance that can be assigned to inflammation as it directly affects the neuron. The fact that we are able to see decreased pain behavior while only a subset of the neuronal cultures demonstrated signal inhibition with the peptide is encouraging.

Given the importance of the immune system, it would be most beneficial to not completely suppress its function. Rather, identifying methods to contain excess inflammation provide much more appealing targets. This idea of inflammatory signal mitigation has been demonstrated in other pathologies stemming from chronic inflammation. Arthritis (in several manifestations) has seen significant benefits of prohibiting the inflammatory cascade, including the administration of sRAGE or the chemotherapeutic drug oxaliplatin to reduce HMGB1 signaling (Hofmann et al., 2002, Ostberg et al., 2008).

Through this investigation of the adaptor proteins MyD88 and TIRAP, an aspect of nociceptive characterization was revealed. The transient receptor vanilloid 1 (TRPV1) is an important thermoreceptor, and known as a 'pathological receptor' due to its responsivity to inflammatory mediators and noxious stimuli (Devesa et al., 2011). TRPV1 is not a true receptor; it is a cation channel by structure, and important in both pain signaling and modulation as they relate to both chronic and mechanical pain (Caterina et al., 1997, Chien et al., 2007, Ferrari et al., 2010). Through experimentation, it was revealed that the sensory neurons responsive to TLR4-induced hyperactivity did not correlate perfectly with capsaicin activated TRPV1 response.

This neuronal TLR4-induced hyperactivity suggests that nociceptive neurons are not equal, and that a diverse population with specific functions may be necessary for pain signaling. This can be seen with the example of the morphine metabolite, M3G. In an animal model M3G administration resulted in pain behavior (Due et al., 2012a, Due et al., 2014). However, this pain behavior was found to be mechanically-derived; thermal

hypersensitivity was not found in the tested animals (Due et al., 2012a). Given that M3G acts through the TLR4 receptor on neurons that can also possess functional TRPV1 channels, these data suggest that nociception functions in a similar manner to inflammation, with multiple moving parts maintained in balance.

Furthermore, TRPV1 can be greatly affected by the expression of inflammatory mediators in the peripheral nervous system (Devesa et al., 2011). Nerve Growth Factor (NGF) and TNF α are among several of the inflammatory mediators shown to significantly increase the expression of TRPV1 channels on the membrane (Zhang et al., 2005, Hensellek et al., 2007). As a result, this can lead to both hypersensitivity and hyperalgesia. Given the role of TRPV1 and the products of inflammation, another possible feed-forward mechanism can be identified. In the case of HMGB1, continued production of the ligand would lead to inflammatory cytokine production, in turn leading to an upregulation in TRPV1 activation. This increased TRPV1 activity could result in exacerbating existing pain behavior, resulting in the perpetuation of the cycle through the release of more HMGB1 or other proinflammatory mediators. This hypothesis becomes more grounded in fact as pathways linking ion channels to inflammation and immunity continue to be discovered and elucidated (Han and Yi, 2014).

Summary and Conclusions

The shared adaptor protein aspect between RAGE and TLR4 suggests possible crosstalk between receptors. There are indeed several similarities and redundancies present when comparing the functions of both RAGE and TLR4 (Sakaguchi et al., 2011,

Ibrahim et al., 2013). There is also the likely scenario of synergy between RAGE and TLR4, since the receptors initiate cellular chemotaxis and cytokine production respectively (Sorci et al., 2012, Lucas and Maes, 2013). This may further explain their separate but related roles in the mitigation of neuropathic pain. Upon initial release of HMGB1 in the all-thiol form, it binds to RAGE, and signals the need for immune cell migration. As these cells potentially arrive, the free HMGB1 could convert to disulfide HMGB1, binding with TLR4 and producing cytokines, possibly having an even further effect upon TRPV1 and other ion channels.

Although both receptors are integral to processes outside of inflammation as it relates to neuropathic pain, metered use of the inhibition of these pathways may mesh well with the current standard of treatment, opioid analgesics. Although opioids are the most effective clinical treatment for neuropathic pain, they are hampered by adverse effects including opioid-induced hyperalgesia and the development of opioid tolerance within the patient (Li, 2012). A combination approach to treatment allows for the two different targets to shoulder less of the respective burden of neuropathic pain treatment, lowering dosages, adverse effects, all the while increasing efficacy and range of the target population. In the case of opioids, combination approaches with anti-epileptics such as carbamazepine have shown great potential in animal models of neuropathic pain to potentiate the effectiveness of morphine as well as result in opioid sparing (Kozer et al., 2008, Due et al., 2014).

Inflammation is not a simplified process of a few targeted ligands and their respective receptors; it is a complicated and organized response system that involved

communication of multiple cell types from both the immune and nervous systems (Sankowski et al., 2015). Chronic inflammation occurs from imbalance within the inflammatory process. Thusly, therapeutic development for these pathologies needs to focus on re-establishing balance and not the complete blockade of a specific junction in the pathway. Traditional approaches to the therapeutic design for inflammatory pathology has revolved around neutralization of the 'offending' molecular signaling via inhibition of immune cell recruitment and activation, and the blockade of pro-inflammatory ligands (Alessandri et al., 2013). Unfortunately, this approach may be short-sighted in nature. Inflammatory pathology is not the result of nascent inflammation processes, but rather continued signaling resulting in progressive and maladaptive chronic inflammation. Thusly, the focus of therapeutic design needs to be sharply focused on the resolution of inflammation, making it effective for not only specific maladies, but a variety of inflammation-derived diseases (Rossi et al., 2007, Serhan, 2008).

This can be seen by the aforementioned study utilizing decoy peptides in an effort to assert a level of control upon HMGB1-derived inflammatory expression. The resolution of chronic inflammation is not the only benefit of studying pharmacological targeting of the HMGB1 signaling pathways. Many of the pathologies that associated with HMGB1 do not provide clinicians with phenotypic events indicative of severity or development, exemplified by the study of intestinal inflammation, as manifested in Chron's disease and ulcerative colitis. Diagnosis and continued observation is mediated through invasive endoscopic procedures or through imaging studies. Both approaches can result in higher cost of care and risk due to the procedure. However, several groups have demonstrated

that HMGB1 can be utilized as a novel biomarker for intestinal inflammation as it relates to disease (Vitali et al., 2011, Palone et al., 2014).

Using HMGB1 as a biomarker was indicative of not only the presence of inflammatory disease, but also its severity. Samples for testing for HMGB1 levels were from the patients' stool, demonstrating not only a novel diagnostic method, but allowing for development of novel therapeutic modalities. Sepharose beads with bound DNA fragments were shown to both bind and sequester HMGB1 when administered orally to the gastrointestinal tract (Ju et al., 2014). Animal studies revealed improvement in body weight, colonic injury, and a decrease in cytokine circulation, as a result of decreasing the reach of HMGB1 activation (Ju et al., 2014). This further supports that relief of inflammatory pathology is not necessarily brought about by blockade, but rather through careful mitigation in order to restore balance to the inflammatory process.

What may be the most fascinating aspect of HMGB1-mediated inflammation is the effect the molecule has prior to vesicular release into the extracellular milieu. HMGB1 within the cell without insult or injurious stimuli acts to bind alternative DNA structures. However, this behavior goes much further than mimicry of histone functionality. DNA repair is highly important to successful growth and maintenance of an organism. HMGB1-bound genetic adducts have been found to both shield the broken DNA construct from repair and enhance the activation of repair pathways, such as the nucleotide excision repair, mismatch, and base excision repair pathways (Lange and Vasquez, 2009). One of the first studies of HMGB1/DNA interactions demonstrated that adding HMGB1 the cell extracts resulted in repair inhibition of one of two specific cisplatin-derived lesions of DNA

(Huang et al., 1994). In turn, the disulfide form of HMGB1 has also demonstrated a lower level of affinity for cisplatin-modified DNA structures, which suggests further importance of oxidative environment as it pertains to HMGB1 function (Wang et al., 2013).

Although there is no definitive finding, what becomes apparent is the limitation of *in vivo* study of *in vitro* processes. In the case of inflammation, and specifically HMGB1, numerous factors play a role in the function of a signal cascade. Constantly operating out of an *in vivo* culture severely limits the study from eventual translation to any clinical application. This necessitates the approach of simultaneous or coordinated investigation of inflammatory function in order to properly elucidate the signaling pathways at work.

The importance of the enclosed studies can be observed in its efforts to approach disease from both the *in vivo* and *in vitro* system. A large subset of the literature focuses upon the role of HMGB1 as it pertains to a particular receptor or cell-mediated function. However the molecule presents as much more than a metaphorical key; it provides a powerful example for the level of redundancy and efficiency needed for survival. HMGB1 is able to maintain naïve functions in DNA repair, be modified to continue nuclear work or secreted to the cytoplasm or extracellular system, tailored in a time and environment dependent manner to particular receptors. This tailoring allows for signal localization, specific inflammatory mediator production, and is dependent upon synchronization with its different receptors. RAGE and TLR4 employ deception via decoy receptors and signal 'heat sinks', and provide multiple pathways for HMGB1 signal transduction, as seen with the TLR4-mediated TRIF/TRAM and TIRAP/MyD88 cascades.

Chapter 5: Future Directions and Studies

The one guarantee of scientific investigation and pursuit is that there is never an end to the discovery. The enclosed studies raise further questions in several interesting areas, and allow for elucidation of the roles of HMGB1, TLR4, and RAGE both within and outside of the nervous system.

The relationship between sodium channels and innate immune signaling

Ion channels are integral to neuronal activity, specifically in the process of generating and firing action potentials. As a result, any neuronal excitation or episodes of hyperactivity will most likely exhibit a direct link to the actions of ion channels. However, in order for signal propagation to result in neuronal firing via an ion channel, a portion of the signal cascade must be therefore dedicated to ion channel recruitment and priming. This has been investigated to a degree with TIRAP and its relationship to the protein phosphatidylinositol 4,5-bisphosphonate (PIP2) (Liu et al., 2010). PIP2 is thought to be important to not only recruitment of TIRAP to the cell membrane, but also in the control of multiple channel proteins (Santos-Sierra et al., 2009). Succinctly, TIRAP and potentially MyD88 may play a larger role in the signal cascades of both RAGE and TLR4, as they may be important to relaying ligand-binding of these receptors to the appropriate channel proteins as well as activating NF- κ B response or another downstream signaling event.

The sodium channels expressed on peripheral neurons became an interest in pain signaling after several clinical studies revealed the efficacy of a general sodium channel blocker, lidocaine, to reduce pain symptomology in patients. These findings were without

severe adverse effects, and purported investigation of not only combinatorial therapeutics, but also deciphering of the signaling pathway responsible (Hutson et al., 2014, Stavropoulou et al., 2014).

Literature evidence demonstrates the connection between TLR function and TRPV1 activation (Diogenes et al., 2011a). However the enclosed studies suggest that the reach of TLR4 signaling in particular may involve more diversity than just the cation channels of the TRP family. Nociceptive activity in the PNS demonstrates a strong correlation to the function of sodium channels (Cummins et al., 2007). Thusly, the pharmacologic targeting of these suspecting sodium channels in the peripheral neuronal system, may be a beneficial method of TLR4 signal mitigation (Due et al., 2012a). A striking example can be found with the anti-epileptic drug (AED) carbamazepine, one of several AEDs that possess a mechanism of action involving blocking the function of sodium channels, including NaV1.7, as it relates to the LPS-TLR4 signaling mechanism (Due et al., 2014). It would particularly interesting to investigate the possible relationship of the same HMGB1-RAGE axis to ion channel functionality, as this presents another method of pharmacological pursuit similar to TLR4.

RAGE in traumatic brain injury

RAGE is not a protein expressed at high levels outside of pathology, with the exception to this rule being the lung, where RAGE is expressed at significantly higher levels than any other tissue (Lopez-Diez et al., 2013). Only one splice variant is predominately found in the lung besides the full-length RAGE, which lacks the

transmembrane domain coding and results in the production of soluble RAGE. This suggests that there is a significant reason for the presence of the RAGE protein in the naïve lung, possibly to act as a PRR or to detect endogenous danger ligands, such as HMGB1. In the pathological event of TBI, RAGE expression was showed to increase in the brain, with HMGB1 expression fluctuating in a pattern mimicking a trend of signal transduction (Gao et al., 2012).

This research topic was investigated by the White laboratory to a high level of success; through collaboration has identified a successful model of traumatic brain injury (TBI) through a controlled cortical impact directly to cortical tissue after craniotomy (Weber et al., 2014). This TBI model allowed for experimentation that allowed for the conclusion that the HMGB1-RAGE axis plays a role in the lung dysfunction initiated by TBI, and that interference with this pathway could possibly result in clinical translation in order to better organ recipient outcomes. Another group has demonstrated the importance of HMGB1-RAGE interaction to the facilitation of TBI, through impeding the binding of HMGB1 to RAGE via the administration of glycyrrhizin (Okuma et al., 2014).

To further this work, efforts would continue to identify the working relationship of HMGB1 and RAGE as the pair relate to acute lung injury pathology as a result of TBI. Through co-immunoprecipitation techniques, as well as other biochemical assays, the relationship between RAGE and the adaptor proteins MyD88 and TIRAP could be investigated within lung tissue, to better understand the signal cascade that results in the TBI-lung injury axis of pathology described within the literature (Weber et al., 2014).

Other adaptor proteins of RAGE could also be studied such as the aforementioned Dia-1, as well as different ligands of RAGE signaling like the S100 family of proteins.

The role of gender in the study of pain and TLR4

The topic of pain and gender is interesting to say the least. Studies have demonstrated that gender can play a role in the development and manifestation of pain behavior in animals. These studies have been bolstered by findings within clinical patient populations regarding differences in disease pathology and overall outcomes as a result of hormonal production and signaling. Bias may be included in more studies than first realized, given that a substantial amount of cell and tissue cultures utilize the phenol red, a compound that can partially mimic estrogenic stimulation; when compared to media free of phenol red, proliferation and progesterone receptor content both increased in cells grown in the presence of phenol red (Berthois et al., 1986). This effect may be minimal upon experimental design; however it represents how the aspect of gender can be subtly overlooked in data interpretation.

Further evidence of gender's role in pathology includes the absence of multiple sclerosis (MS) relapses in patients during pregnancy, thought to be due to manipulated levels of circulating sex steroids (Garay et al., 2007). In the animal model of MS (autoimmune encephalomyelitis mice (EAE)) it was shown that the TLR4 receptor levels were increased, and that TLR4 was down-regulated upon progesterone administration (Garay et al., 2012). This progesterone administration may prevent the activation of pro-inflammatory genes and cytokines (such as IL-2, IL-17, and TNF α) downstream of TLR4

signaling as well as oligodendrocyte death and axonal demyelination in EAE mice (De Nicola et al., 2013).

With regards to chronic pain manifestation clinically, female patients have shown to be more likely to experience chronic pain when compared to male patients, as well as experience pain of greater severity and duration (Gintzler and Liu, 2012). It has also been observed that female patients often report lower thresholds to experimental stimuli as compared with men in pain studies (Lacroix-Fralish et al., 2006). Intriguingly, women have been reported to consume significantly less morphine in situations of patient-controlled analgesia three days post-operation compared to men, making gender one of the strongest predictors of post-operative morphine requirements (Chia et al., 2002). An animal model of long-term ovariectomy in adult female rats demonstrates induction of thermal and mechanical hyperalgesia that can be reversed by estradiol replacement. In addition to the finding of functional estrogen receptors in the murine dorsal root ganglia, altogether this portrays a potentially impactful role for gender in both inflammatory-derived pathology (Gintzler and Liu, 2012). Significant differences have been found between the genders in rats, specifically relating to their expression of TLR4 (Sorge et al., 2011).

Our model of neuropathic pain via tibial nerve ligation could serve as an interesting model for gender pain studies. Combining the TNI with gonadectomy, along with the variables of hormone treatments and the decoy peptides used successfully in the aforementioned experiments, could provide valuable insight to the role of gender in inflammatory signaling and pain behavior.

HMGB1 and RAGE in cellular repair, proliferation and cancer

It is important to continue to define the role of HMGB1 in pathology due to its overexpression in cancerous tissue. This HMGB1 overexpression has been observed in both breast and colon cancer, which have a clinical footprint of over 300,000 new cases annually (ACS, 2015). The aforementioned ability of HMGB1 to interact with TFs and enhance their activation is of particular interest, given that several of these factors are well known for their functions in cancer biology.

The binding of all-thiol HMGB1 to the RAGE receptor has been linked to oncological events, including tumor growth and metastasis (Ellerman et al., 2007). These events are often observed in correlation with both HMGB1 and RAGE overexpression, which suggests a large role of HMGB1/RAGE signaling within cancer biology and signaling (Fahmueller et al., 2012). THE RAGE protein has been demonstrated to increase resistance to chemotherapeutic modalities and to improve survival and inhibit apoptosis by limiting the ability of p53 (which acts as a tumor suppressor) to translocate to the mitochondria (Kang et al., 2010). Under genetic knockdown (KO) of expression, the absence of RAGE has been shown to be responsible for a 40 to 70% decline in anti-apoptotic activity via such proteins as Bcl-2 and Bcl-XL (Kang et al., 2010). These RAGE KO animals were overall less susceptible to both acute inflammatory damage and carcinogen-derived tumor development (Kang et al., 2010). Another study concluded that autophagy induced by RAGE activation is potentially integral to early metastasis of disease in a model of pancreatic cancer (Xie et al., 2013).

The release of HMGB1 (with a higher level of RAGE affinity immediately following release) has been observed to be constitutive in mesothelioma and other cancerous cells (Venereau et al., 2012a). High levels of HMGB1 were also observed in the serum of patients with colorectal cancer (Fahmueller et al., 2012). The nascent role of HMGB1 in the binding of alternative DNA structures is also relevant. HMGB1 has a significantly higher binding affinity to cisplatin damaged DNA over other varieties of DNA bends and breaks. Platinum-based chemotherapeutic drugs typically produce two DNA adducts, both of which are repairable through the NER system (Huang et al., 1994). However, HMGB1 binding of these adducts has been demonstrated to be one of the proponents of the aforementioned 'repair shielding' theory, and possible cellular resistance to chemotherapy (Nagatani et al., 2001, Mitkova et al., 2005).

It would be interesting to see if the same mechanisms that appear to play a significant role in HMGB1/RAGE signaling in chronic pain and neuroinflammation affect cancer biology and signaling. The decoy peptides used to mediate pain behavior in the animal pain model and neuronal hyperactivity *in vitro* may reveal new targets for chemotherapy, as well as provide a better understanding in the oncologic pathway of HMGB1 and RAGE.

Appendix

Decoy Peptide Concentration Analysis:

<u>Concentration of Decoy Peptide</u>	<u>Cell Responses</u>
5mM	0
10mM	0
15mM	0
20mM	22
25mM	0
30mM	20
35mM	5
40mM	16
45mM	1
50mM	5

References

- (1992) Acute pain management in infants, children and adolescents: operative and medical procedures. Agency for Health Care Policy and Research. American family physician 46:469-479.
- Abolfathi Momtaz Y, Ibrahim R, Hamid TA (2013) The impact of giving support to others on older adults' perceived health status. Psychogeriatrics : the official journal of the Japanese Psychogeriatric Society.
- Acosta C, Davies A (2008) Bacterial lipopolysaccharide regulates nociceptin expression in sensory neurons. Journal of neuroscience research 86:1077-1086.
- ACS (2015) Cancer Facts & Figures 2015. Atlanta: American Cancer Society.
- Agresti A, Lupo R, Bianchi ME, Muller S (2003) HMGB1 interacts differentially with members of the Rel family of transcription factors. Biochemical and biophysical research communications 302:421-426.
- Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124:783-801.
- Akirav EM, Preston-Hurlburt P, Garyu J, Henegariu O, Clynes R, Schmidt AM, Herold KC (2012) RAGE expression in human T cells: a link between environmental factors and adaptive immune responses. PLoS One 7:e34698.
- Alessandri AL, Sousa LP, Lucas CD, Rossi AG, Pinho V, Teixeira MM (2013) Resolution of inflammation: mechanisms and opportunity for drug development. Pharmacology & therapeutics 139:189-212.

- Allette YM, Due MR, Wilson SM, Feldman P, Ripsch MS, Khanna R, White FA (2014) Identification of a functional interaction of HMGB1 with Receptor for Advanced Glycation End-products in a model of neuropathic pain. *Brain, behavior, and immunity* 42:169-177.
- Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ (2002) HMGB1 as a DNA-binding cytokine. *Journal of leukocyte biology* 72:1084-1091.
- Andersson U, Tracey KJ (2011) HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol* 29:139-162.
- Andreaskos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, Monaco C, Feldmann M, Foxwell BM (2004) Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood* 103:2229-2237.
- Aneja RK, Tsung A, Sjodin H, Geftter JV, Delude RL, Billiar TR, Fink MP (2008) Preconditioning with high mobility group box 1 (HMGB1) induces lipopolysaccharide (LPS) tolerance. *Journal of leukocyte biology* 84:1326-1334.
- Antoine DJ, Williams DP, Kipar A, Lavery H, Park BK (2010) Diet restriction inhibits apoptosis and HMGB1 oxidation and promotes inflammatory cell recruitment during acetaminophen hepatotoxicity. *Mol Med* 16:479-490.
- Asmundson GJ, Norton GR, Jacobson SJ (1996) Social, blood/injury, and agoraphobic fears in patients with physically unexplained chronic pain: are they clinically significant? *Anxiety* 2:28-33.

- Assenberg R, Webb M, Connolly E, Stott K, Watson M, Hobbs J, Thomas JO (2008) A critical role in structure-specific DNA binding for the acetyltable lysine residues in HMGB1. *The Biochemical journal* 411:553-561.
- Azad N, Rojanasakul Y, Vallyathan V (2008) Inflammation and lung cancer: roles of reactive oxygen/nitrogen species. *Journal of toxicology and environmental health Part B, Critical reviews* 11:1-15.
- Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. *Annual review of immunology* 12:141-179.
- Balermipas P, Bauer C, Fraunholz I, Ottinger A, Wagenblast J, Stover T, Seitz O, Fokas E, Rodel C, Weiss C (2014) Concomitant chemoradiotherapy versus induction chemotherapy followed by chemoradiotherapy as definitive, first line treatment of squamous cell carcinoma of the head and neck. A retrospective single center analysis. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft [et al]* 190:256-262.
- Bansal S, Chawla D, Banerjee BD, Madhu SV, Tripathi AK (2013) Association of RAGE gene polymorphism with circulating AGEs level and paraoxonase activity in relation to macro-vascular complications in Indian type 2 diabetes mellitus patients. *Gene* 526:325-330.
- Barton GM, Medzhitov R (2002) Toll-like receptors and their ligands. *Current topics in microbiology and immunology* 270:81-92.

- Bell CW, Jiang W, Reich CF, 3rd, Pisetsky DS (2006) The extracellular release of HMGB1 during apoptotic cell death. *American journal of physiology Cell physiology* 291:C1318-1325.
- Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America* 83:2496-2500.
- Bevan DE, Martinko AJ, Loram LC, Stahl JA, Taylor FR, Joshee S, Watkins LR, Yin H (2010) Selection, Preparation, and Evaluation of Small- Molecule Inhibitors of Toll-Like Receptor 4. *ACS medicinal chemistry letters* 1:194-198.
- Bhangoo S, Ren D, Miller RJ, Henry KJ, Lineswala J, Hamdouchi C, Li B, Monahan PE, Chan DM, Ripsch MS, White FA (2007) Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. *Mol Pain* 3:38.
- Bianchi ME (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of leukocyte biology* 81:1-5.
- Bianchi ME, Falciola L, Ferrari S, Lilley DM (1992) The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins. *EMBO J* 11:1055-1063.

- Bianchi ME, Manfredi AA (2007) High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunological reviews* 220:35-46.
- Bianchi R, Kastrisianaki E, Giambanco I, Donato R (2011) S100B protein stimulates microglia migration via RAGE-dependent up-regulation of chemokine expression and release. *The Journal of biological chemistry* 286:7214-7226.
- Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM, Nawroth PP (2005) Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med (Berl)* 83:876-886.
- Binshok AM, Wang H, Zimmermann K, Amaya F, Vardeh D, Shi L, Brenner GJ, Ji RR, Bean BP, Woolf CJ, Samad TA (2008) Nociceptors are interleukin-1beta sensors. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28:14062-14073.
- Black JA, Liu S, Waxman SG (2009) Sodium channel activity modulates multiple functions in microglia. *Glia* 57:1072-1081.
- Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *The EMBO journal* 22:5551-5560.
- Bork P, Holm L, Sander C (1994) The immunoglobulin fold. Structural classification, sequence patterns and common core. *Journal of molecular biology* 242:309-320.
- Brittain JM, Duarte DB, Wilson SM, Zhu W, Ballard C, Johnson PL, Liu N, Xiong W, Ripsch MS, Wang Y, Fehrenbacher JC, Fitz SD, Khanna M, Park CK, Schmutzler BS, Cheon

- BM, Due MR, Brustovetsky T, Ashpole NM, Hudmon A, Meroueh SO, Hingtgen CM, Brustovetsky N, Ji RR, Hurley JH, Jin X, Shekhar A, Xu XM, Oxford GS, Vasko MR, White FA, Khanna R (2011) Suppression of inflammatory and neuropathic pain by uncoupling CRMP-2 from the presynaptic Ca²⁺(+) channel complex. *Nat Med* 17:822-829.
- Brune B, Dehne N, Grossmann N, Jung M, Namgaladze D, Schmid T, von Knethen A, Weigert A (2013) Redox control of inflammation in macrophages. *Antioxidants & redox signaling* 19:595-637.
- Bsibsi M, Ravid R, Gveric D, van Noort JM (2002) Broad expression of Toll-like receptors in the human central nervous system. *Journal of neuropathology and experimental neurology* 61:1013-1021.
- Bucciarelli LG, Kaneko M, Ananthakrishnan R, Harja E, Lee LK, Hwang YC, Lerner S, Bakr S, Li Q, Lu Y, Song F, Qu W, Gomez T, Zou YS, Yan SF, Schmidt AM, Ramasamy R (2006) Receptor for advanced-glycation end products: key modulator of myocardial ischemic injury. *Circulation* 113:1226-1234.
- Buchanan MM, Hutchinson M, Watkins LR, Yin H (2010) Toll-like receptor 4 in CNS pathologies. *Journal of neurochemistry* 114:13-27.
- Buckley ST, Ehrhardt C (2010) The receptor for advanced glycation end products (RAGE) and the lung. *Journal of biomedicine & biotechnology* 2010:917108.
- Bustin M (2001) Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends in biochemical sciences* 26:152-153.

- Calogero S, Grassi F, Aguzzi A, Voigtlander T, Ferrier P, Ferrari S, Bianchi ME (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nature genetics* 22:276-280.
- Calvo M, Dawes JM, Bennett DL (2012) The role of the immune system in the generation of neuropathic pain. *Lancet Neurol* 11:629-642.
- Campana L, Bosurgi L, Bianchi ME, Manfredi AA, Rovere-Querini P (2009) Requirement of HMGB1 for stromal cell-derived factor-1/CXCL12-dependent migration of macrophages and dendritic cells. *J Leukoc Biol* 86:609-615.
- Candeias SM, Testard I (2015) The many interactions between the innate immune system and the response to radiation. *Cancer letters*.
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816-824.
- Chacur M, Milligan ED, Gazda LS, Armstrong C, Wang H, Tracey KJ, Maier SF, Watkins LR (2001) A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. *Pain* 94:231-244.
- Chandrasoma PT, Clive R. (1997) *Concise Pathology* Appleton & Lange.
- Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, Nagashima M, Morser J, Arnold B, Preissner KT, Nawroth PP (2003) The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for

- inflammatory cell recruitment. *The Journal of experimental medicine* 198:1507-1515.
- Chen G, Cai L, Chen B, Liang J, Lin F, Li L, Lin L, Yao J, Wen J, Huang H (2011) Serum level of endogenous secretory receptor for advanced glycation end products and other factors in type 2 diabetic patients with mild cognitive impairment. *Diabetes care* 34:2586-2590.
- Chen GY, Nunez G (2010) Sterile inflammation: sensing and reacting to damage. *Nature reviews Immunology* 10:826-837.
- Cheng K, Wang X, Zhang S, Yin H (2012) Discovery of Small-Molecule Inhibitors of the TLR1/TLR2 Complex. *Angew Chem Int Ed Engl*.
- Chia YY, Chow LH, Hung CC, Liu K, Ger LP, Wang PN (2002) Gender and pain upon movement are associated with the requirements for postoperative patient-controlled iv analgesia: a prospective survey of 2,298 Chinese patients. *Canadian journal of anaesthesia = Journal canadien d'anesthesie* 49:249-255.
- Chiavegato A, Zurolo E, Losi G, Aronica E, Carmignoto G (2014) The inflammatory molecules IL-1 β and HMGB1 can rapidly enhance focal seizure generation in a brain slice model of temporal lobe epilepsy. *Frontiers in cellular neuroscience* 8:155.
- Chien LY, Cheng JK, Chu D, Cheng CF, Tsaur ML (2007) Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27:9855-9865.

- Chiu IM, Heesters BA, Ghasemlou N, Von Hehn CA, Zhao F, Tran J, Wainger B, Strominger A, Muralidharan S, Horswill AR, Bubeck Wardenburg J, Hwang SW, Carroll MC, Woolf CJ (2013) Bacteria activate sensory neurons that modulate pain and inflammation. *Nature* 501:52-57.
- Choy M, Dube CM, Patterson K, Barnes SR, Maras P, Blood AB, Hasso AN, Obenaus A, Baram TZ (2014) A novel, noninvasive, predictive epilepsy biomarker with clinical potential. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 34:8672-8684.
- Chung KF (2012) Inflammatory biomarkers in severe asthma. *Current opinion in pulmonary medicine* 18:35-41.
- Conti E, Uy M, Leighton L, Blobel G, Kuriyan J (1998) Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. *Cell* 94:193-204.
- Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 17:1195-1214.
- Crombez G, Vlaeyen JW, Heuts PH, Lysens R (1999) Pain-related fear is more disabling than pain itself: evidence on the role of pain-related fear in chronic back pain disability. *Pain* 80:329-339.
- Cummins TR, Sheets PL, Waxman SG (2007) The roles of sodium channels in nociception: Implications for mechanisms of pain. *Pain* 131:243-257.

- Cunha FQ, Poole S, Lorenzetti BB, Ferreira SH (1992) The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. *British journal of pharmacology* 107:660-664.
- Czura CJ, Wang H, Tracey KJ (2001) Dual roles for HMGB1: DNA binding and cytokine. *J Endotoxin Res* 7:315-321.
- Dattilo BM, Fritz G, Leclerc E, Kooi CW, Heizmann CW, Chazin WJ (2007) The extracellular region of the receptor for advanced glycation end products is composed of two independent structural units. *Biochemistry* 46:6957-6970.
- Davis CN, Mann E, Behrens MM, Gaidarova S, Rebek M, Rebek J, Jr., Bartfai T (2006) MyD88-dependent and -independent signaling by IL-1 in neurons probed by bifunctional Toll/IL-1 receptor domain/BB-loop mimetics. *Proc Natl Acad Sci U S A* 103:2953-2958.
- De Nicola AF, Gonzalez-Deniselle MC, Garay L, Meyer M, Gargiulo-Monachelli G, Guennoun R, Schumacher M, Carreras MC, Poderoso JJ (2013) Progesterone Protective Effects In Neurodegeneration And Neuroinflammation. *Journal of neuroendocrinology*.
- Devesa I, Planells-Cases R, Fernandez-Ballester G, Gonzalez-Ros JM, Ferrer-Montiel A, Fernandez-Carvajal A (2011) Role of the transient receptor potential vanilloid 1 in inflammation and sepsis. *Journal of inflammation research* 4:67-81.
- Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM (2011a) LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *Journal of dental research* 90:759-764.

- Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM (2011b) LPS Sensitizes TRPV1 via Activation of TLR4 in Trigeminal Sensory Neurons. *J Dent Res* Jun;90(6):759-764.
- Donato R (2007) RAGE: a single receptor for several ligands and different cellular responses: the case of certain S100 proteins. *Current molecular medicine* 7:711-724.
- Drew LJ, Wood JN, Cesare P (2002) Distinct mechanosensitive properties of capsaicin-sensitive and -insensitive sensory neurons. *J Neurosci* 22:RC228.
- Du H, Li P, Wang J, Qing X, Li W (2012) The interaction of amyloid beta and the receptor for advanced glycation endproducts induces matrix metalloproteinase-2 expression in brain endothelial cells. *Cellular and molecular neurobiology* 32:141-147.
- Due MR, Piekarz AD, Wilson N, Feldman P, Ripsch MS, Chavez S, Yin H, Khanna R, White FA (2012a) Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *Journal of neuroinflammation* 9:200.
- Due MR, Piekarz AD, Wilson N, Feldman P, Ripsch MS, Chavez S, Yin H, Khanna R, White FA (2012b) Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *J Neuroinflammation* 9:200.
- Due MR, Yang XF, Allette YM, Randolph AL, Ripsch MS, Wilson SM, Dustrude ET, Khanna R, White FA (2014) Carbamazepine potentiates the effectiveness of morphine in a rodent model of neuropathic pain. *PloS one* 9:e107399.

- Dumitriu IE, Baruah P, Manfredi AA, Bianchi ME, Rovere-Querini P (2005) HMGB1: guiding immunity from within. *Trends Immunol* 26:381-387.
- Dzau VJ, Pizzo PA (2014) Relieving pain in America: insights from an Institute of Medicine committee. *Jama* 312:1507-1508.
- Eccleston C (2001) Role of psychology in pain management. *British journal of anaesthesia* 87:144-152.
- Eidson LN, Murphy AZ (2013) Blockade of Toll-like receptor 4 attenuates morphine tolerance and facilitates the pain relieving properties of morphine. *J Neurosci* 33:15952-15963.
- Elenkov I, Pelovsky P, Ugrinova I, Takahashi M, Pasheva E (2011) The DNA binding and bending activities of truncated tail-less HMGB1 protein are differentially affected by Lys-2 and Lys-81 residues and their acetylation. *International journal of biological sciences* 7:691-699.
- Ellerman JE, Brown CK, de Vera M, Zeh HJ, Billiar T, Rubartelli A, Lotze MT (2007) Masquerader: high mobility group box-1 and cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 13:2836-2848.
- Ely EW, Shintani A, Truman B, Speroff T, Gordon SM, Harrell FE, Jr., Inouye SK, Bernard GR, Dittus RS (2004) Delirium as a predictor of mortality in mechanically ventilated patients in the intensive care unit. *Jama* 291:1753-1762.
- Erridge C (2010) Endogenous ligands of TLR2 and TLR4: agonists or assistants? *Journal of leukocyte biology* 87:989-999.

- Fages C, Nolo R, Huttunen HJ, Eskelinen E, Rauvala H (2000) Regulation of cell migration by amphotericin. *J Cell Sci* 113 (Pt 4):611-620.
- Fahmueller YN, Nagel D, Hoffmann RT, Tatsch K, Jakobs T, Stieber P, Holdenrieder S (2012) Immunogenic cell death biomarkers HMGB1, RAGE and DNase indicate response to radioembolisation therapy and prognosis in colorectal cancer patients. *International journal of cancer Journal international du cancer*.
- Fekonja O, Bencina M, Jerala R (2012) Toll/interleukin-1 receptor domain dimers as the platform for activation and enhanced inhibition of Toll-like receptor signaling. *The Journal of biological chemistry* 287:30993-31002.
- Feldman P, Due MR, Ripsch MS, Khanna R, White FA (2012) The persistent release of HMGB1 contributes to tactile hyperalgesia in a rodent model of neuropathic pain. *J Neuroinflammation* 9:180.
- Ferrari LF, Bogen O, Levine JD (2010) Nociceptor subpopulations involved in hyperalgesic priming. *Neuroscience* 165:896-901.
- Fitzner N, Clauberg S, Essmann F, Liebmann J, Kolb-Bachofen V (2008) Human skin endothelial cells can express all 10 TLR genes and respond to respective ligands. *Clinical and vaccine immunology : CVI* 15:138-146.
- Franchi S, Moretti S, Castelli M, Lattuada D, Scavullo C, Panerai AE, Sacerdote P (2012) Mu opioid receptor activation modulates Toll like receptor 4 in murine macrophages. *Brain, behavior, and immunity* 26:480-488.

- Gao TL, Yuan XT, Yang D, Dai HL, Wang WJ, Peng X, Shao HJ, Jin ZF, Fu ZJ (2012) Expression of HMGB1 and RAGE in rat and human brains after traumatic brain injury. *The journal of trauma and acute care surgery* 72:643-649.
- Garay L, Deniselle MC, Lima A, Roig P, De Nicola AF (2007) Effects of progesterone in the spinal cord of a mouse model of multiple sclerosis. *The Journal of steroid biochemistry and molecular biology* 107:228-237.
- Garay LI, Gonzalez Deniselle MC, Brocca ME, Lima A, Roig P, De Nicola AF (2012) Progesterone down-regulates spinal cord inflammatory mediators and increases myelination in experimental autoimmune encephalomyelitis. *Neuroscience* 226:40-50.
- Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, Rubartelli A (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO reports* 3:995-1001.
- Gebhardt C, Riehl A, Durchdewald M, Nemeth J, Furstenberger G, Muller-Decker K, Enk A, Arnold B, Bierhaus A, Nawroth PP, Hess J, Angel P (2008) RAGE signaling sustains inflammation and promotes tumor development. *The Journal of experimental medicine* 205:275-285.
- George J, Kubarenko AV, Rautanen A, Mills TC, Colak E, Kempf T, Hill AV, Nieters A, Weber AN (2010) MyD88 adaptor-like D96N is a naturally occurring loss-of-function variant of TIRAP. *J Immunol* 184:3025-3032.

- Giese K, Cox J, Grosschedl R (1992) The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell* 69:185-195.
- Gintzler AR, Liu NJ (2012) Importance of sex to pain and its amelioration; relevance of spinal estrogens and its membrane receptors. *Frontiers in neuroendocrinology* 33:412-424.
- Gong CY, Zhou AL, Mao JH, Hu YE, Geng JS (2014) The role of Toll-like receptor 4 on inflammation and Abeta formation in cortex astrocytes. *Sheng li xue bao : [Acta physiologica Sinica]* 66:631-638.
- Grace PM, Ramos KM, Rodgers KM, Wang X, Hutchinson MR, Lewis MT, Morgan KN, Kroll JL, Taylor FR, Strand KA, Zhang Y, Berkelhammer D, Huey MG, Greene LI, Cochran TA, Yin H, Barth DS, Johnson KW, Rice KC, Maier SF, Watkins LR (2014) Activation of adult rat CNS endothelial cells by opioid-induced toll-like receptor 4 (TLR4) signaling induces proinflammatory, biochemical, morphological, and behavioral sequelae. *Neuroscience* 280:299-317.
- Guo J, Hsieh C, Wu Z, DiGiammarino EL, Luo F, Fox GB, Harlan JE, Schmidt M, Loebbert R, Mueller R, Ebert U, Nimmrich V (2012) Antibodies to receptor of advanced glycation end products (RAGE) and uses thereof. (Office, U. S. P. a. T., ed) United States: Abbott Laboratories, Abbott Park, IL Abbott GmbH & Co. KG, Wiesbaden-Delkenheim, DE.
- Hallman M, Ramet M, Ezekowitz RA (2001) Toll-like receptors as sensors of pathogens. *Pediatric research* 50:315-321.

- Han H, Yi F (2014) New insights into TRP channels: Interaction with pattern recognition receptors. *Channels* 8:13-19.
- Hasselstrom J, Sawe J (1993) Morphine pharmacokinetics and metabolism in humans. Enterohepatic cycling and relative contribution of metabolites to active opioid concentrations. *Clinical pharmacokinetics* 24:344-354.
- Heap GA, van Heel DA (2009) The genetics of chronic inflammatory diseases. *Human molecular genetics* 18:R101-106.
- Hensellek S, Brell P, Schaible HG, Brauer R, Segond von Banchet G (2007) The cytokine TNFalpha increases the proportion of DRG neurones expressing the TRPV1 receptor via the TNFR1 receptor and ERK activation. *Molecular and cellular neurosciences* 36:381-391.
- Hofmann MA, Drury S, Hudson BI, Gleason MR, Qu W, Lu Y, Lalla E, Chitnis S, Monteiro J, Stickland MH, Bucciarelli LG, Moser B, Moxley G, Itescu S, Grant PJ, Gregersen PK, Stern DM, Schmidt AM (2002) RAGE and arthritis: the G82S polymorphism amplifies the inflammatory response. *Genes and immunity* 3:123-135.
- Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H (1999) MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 99:81-92.
- Hoppe G, Talcott KE, Bhattacharya SK, Crabb JW, Sears JE (2006) Molecular basis for the redox control of nuclear transport of the structural chromatin protein Hmgb1. *Experimental cell research* 312:3526-3538.

- Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, et al. (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *The Journal of biological chemistry* 270:25752-25761.
- Horng T, Barton GM, Medzhitov R (2001) TIRAP: an adapter molecule in the Toll signaling pathway. *Nature immunology* 2:835-841.
- Horng T, Medzhitov R (2001) *Drosophila* MyD88 is an adapter in the Toll signaling pathway. *Proc Natl Acad Sci U S A* 98:12654-12658.
- Hou L, Wang X (2001) PKC and PKA, but not PKG mediate LPS-induced CGRP release and $[Ca^{2+}]_i$ elevation in DRG neurons of neonatal rats. *J Neurosci Res* 66:592-600.
- Hreggvidsdottir HS, Lundberg AM, Aveberger AC, Klevenvall L, Andersson U, Harris HE (2012) High mobility group box protein 1 (HMGB1)-partner molecule complexes enhance cytokine production by signaling through the partner molecule receptor. *Mol Med* 18:224-230.
- Hua XY, Chen P, Fox A, Myers RR (1996) Involvement of cytokines in lipopolysaccharide-induced facilitation of CGRP release from capsaicin-sensitive nerves in the trachea: studies with interleukin-1 β and tumor necrosis factor- α . *J Neurosci* 16:4742-4748.
- Huang JC, Zamble DB, Reardon JT, Lippard SJ, Sancar A (1994) HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug

- cisplatin by human excision nuclease. *Proceedings of the National Academy of Sciences of the United States of America* 91:10394-10398.
- Hudson BI, Kalea AZ, Del Mar Arriero M, Harja E, Boulanger E, D'Agati V, Schmidt AM (2008) Interaction of the RAGE cytoplasmic domain with diaphanous-1 is required for ligand-stimulated cellular migration through activation of Rac1 and Cdc42. *The Journal of biological chemistry* 283:34457-34468.
- Hutchinson MR, Shavit Y, Grace PM, Rice KC, Maier SF, Watkins LR (2011) Exploring the neuroimmunopharmacology of opioids: an integrative review of mechanisms of central immune signaling and their implications for opioid analgesia. *Pharmacol Rev* 63:772-810.
- Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, Slivka PF, Coats BD, Rezvani N, Wieseler J, Hughes TS, Landgraf KE, Chan S, Fong S, Phipps S, Falke JJ, Leinwand LA, Maier SF, Yin H, Rice KC, Watkins LR (2010) Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain, behavior, and immunity* 24:83-95.
- Hutson P, Backonja M, Knurr H (2014) Intravenous Lidocaine for Neuropathic Pain: A Retrospective Analysis of Tolerability and Efficacy. *Pain Med*.
- Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H (2002) Receptor for advanced glycation end products-binding COOH-terminal motif of amphotericin inhibits invasive migration and metastasis. *Cancer Res* 62:4805-4811.
- Huttunen HJ, Fages C, Rauvala H (1999) Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the

cytoplasmic domain of the receptor but different downstream signaling pathways.

The Journal of biological chemistry 274:19919-19924.

Ibrahim ZA, Armour CL, Phipps S, Sukkar MB (2013) RAGE and TLRs: relatives, friends or neighbours? Molecular immunology 56:739-744.

Iori V, Maroso M, Rizzi M, Iyer AM, Vertemara R, Carli M, Agresti A, Antonelli A, Bianchi ME, Aronica E, Ravizza T, Vezzani A (2013) Receptor for Advanced Glycation Endproducts is upregulated in temporal lobe epilepsy and contributes to experimental seizures. Neurobiology of disease 58:102-114.

Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. Nature 461:1071-1078.

Janeway CA, Jr., Medzhitov R (2002) Innate immune recognition. Annual review of immunology 20:197-216.

Jayaraman L, Moorthy NC, Murthy KG, Manley JL, Bustin M, Prives C (1998) High mobility group protein-1 (HMG-1) is a unique activator of p53. Genes & development 12:462-472.

Ji H, Rabbi MF, Labis B, Pavlov VA, Tracey KJ, Ghia JE (2014a) Central cholinergic activation of a vagus nerve-to-spleen circuit alleviates experimental colitis. Mucosal immunology 7:335-347.

Ji RR, Xu ZZ, Gao YJ (2014b) Emerging targets in neuroinflammation-driven chronic pain. Nature reviews Drug discovery 13:533-548.

Jiao Y, Wang HC, Fan SJ (2007) Growth suppression and radiosensitivity increase by HMGB1 in breast cancer. Acta pharmacologica Sinica 28:1957-1967.

- Jin MS, Lee JO (2008) Structures of the toll-like receptor family and its ligand complexes. *Immunity* 29:182-191.
- Joshi SR, Sarpong YC, Peterson RC, Scovell WM (2012) Nucleosome dynamics: HMGB1 relaxes canonical nucleosome structure to facilitate estrogen receptor binding. *Nucleic acids research* 40:10161-10171.
- Ju Z, Chavan SS, Antoine DJ, Dancho M, Tsaava T, Li J, Lu B, Levine YA, Stiegler A, Tamari Y, Al-Abed Y, Roth J, Tracey KJ, Yang H (2014) Sequestering HMGB1 via DNA-conjugated beads ameliorates murine colitis. *PloS one* 9:e103992.
- Jules J, Maiguel D, Hudson BI (2013) Alternative splicing of the RAGE cytoplasmic domain regulates cell signaling and function. *PloS one* 8:e78267.
- Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125:943-955.
- Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 9:361-368.
- Kalea AZ, Reiniger N, Yang H, Arriero M, Schmidt AM, Hudson BI (2009) Alternative splicing of the murine receptor for advanced glycation end-products (RAGE) gene. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23:1766-1774.
- Kammeyer A, Luiten RM (2015) Oxidation events and skin aging. *Ageing research reviews* 21C:16-29.
- Kang R, Tang D, Schapiro NE, Livesey KM, Farkas A, Loughran P, Bierhaus A, Lotze MT, Zeh HJ (2010) The receptor for advanced glycation end products (RAGE) sustains

autophagy and limits apoptosis, promoting pancreatic tumor cell survival. *Cell death and differentiation* 17:666-676.

Karatas H, Erdener SE, Gursay-Ozdemir Y, Lule S, Eren-Kocak E, Sen ZD, Dalkara T (2013) Spreading depression triggers headache by activating neuronal Panx1 channels. *Science* 339:1092-1095.

Katz J, Seltzer Z (2009) Transition from acute to chronic postsurgical pain: risk factors and protective factors. *Expert review of neurotherapeutics* 9:723-744.

Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, Miyake K, Zhang J, Lee WP, Muszynski A, Forsberg LS, Carlson RW, Dixit VM (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341:1246-1249.

Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA (2008) Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 29:21-32.

Keyel PA (2014) How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1. *Cytokine* 69:136-145.

Khor CC, Chapman SJ, Vannberg FO, Dunne A, Murphy C, Ling EY, Frodsham AJ, Walley AJ, Kyrieleis O, Khan A, Aucan C, Segal S, Moore CE, Knox K, Campbell SJ, Lienhardt C, Scott A, Aaby P, Sow OY, Grignani RT, Sillah J, Sirugo G, Peshu N, Williams TN, Maitland K, Davies RJ, Kwiatkowski DP, Day NP, Yala D, Crook DW, Marsh K, Berkley JA, O'Neill LA, Hill AV (2007) A Mal functional variant is associated with

- protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nature genetics* 39:523-528.
- Kierdorf K, Fritz G (2013) RAGE regulation and signaling in inflammation and beyond. *Journal of leukocyte biology*.
- Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, Enkhbayar P, Matsushima N, Lee H, Yoo OJ, Lee JO (2007) Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* 130:906-917.
- Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A (2008) HMGB1: endogenous danger signaling. *Mol Med* 14:476-484.
- Kozer E, Levichek Z, Hoshino N, Kapur B, Leombruno J, Taguchi N, Garcia-Bournissen F, Koren G, Ito S (2008) The effect of amitriptyline, gabapentin, and carbamazepine on morphine-induced hypercarbia in rabbits. *Anesthesia and analgesia* 107:1216-1222.
- Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G, Nomenclature Committee on Cell D (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell death and differentiation* 16:3-11.
- Kumar V, Abbas AK, Fausto N, Robbins SL, Cotran RS (2005) Robbins and Cotran pathologic basis of disease. Philadelphia: Elsevier Saunders.

- Lacroix-Fralish ML, Tawfik VL, Nutile-McMenemy N, DeLeo JA (2006) Progesterone mediates gonadal hormone differences in tactile and thermal hypersensitivity following L5 nerve root ligation in female rats. *Neuroscience* 138:601-608.
- Lamkanfi M, Dixit VM (2009) Inflammasomes: guardians of cytosolic sanctity. *Immunological reviews* 227:95-105.
- Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM (1997) Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *The Journal of biological chemistry* 272:17810-17814.
- Lange SS, Vasquez KM (2009) HMGB1: the jack-of-all-trades protein is a master DNA repair mechanic. *Molecular carcinogenesis* 48:571-580.
- Lee SA, Kwak MS, Kim S, Shin JS (2014) The role of high mobility group box 1 in innate immunity. *Yonsei medical journal* 55:1165-1176.
- Leone S, Silipo A, E LN, Lanzetta R, Parrilli M, Molinaro A (2007) Molecular structure of endotoxins from Gram-negative marine bacteria: an update. *Marine drugs* 5:85-112.
- Leow-Dyke S, Allen C, Denes A, Nilsson O, Maysami S, Bowie AG, Rothwell NJ, Pinteaux E (2012) Neuronal Toll-like receptor 4 signaling induces brain endothelial activation and neutrophil transmigration in vitro. *Journal of neuroinflammation* 9:230.
- Lewis SS, Hutchinson MR, Rezvani N, Loram LC, Zhang Y, Maier SF, Rice KC, Watkins LR (2010) Evidence that intrathecal morphine-3-glucuronide may cause pain

- enhancement via toll-like receptor 4/MD-2 and interleukin-1 β . *Neuroscience* 165:569-583.
- Li Q (2012) Antagonists of toll like receptor 4 maybe a new strategy to counteract opioid-induced hyperalgesia and opioid tolerance. *Medical hypotheses* 79:754-756.
- Li Y, Zhang H, Kosturakis AK, Jawad AB, Dougherty PM (2014) Toll-like receptor 4 signaling contributes to paclitaxel-induced peripheral neuropathy. *J Pain*.
- Liu T, Gao YJ, Ji RR (2012) Emerging role of Toll-like receptors in the control of pain and itch. *Neuroscience bulletin* 28:131-144.
- Liu Y, Li M, Fan S, Lin Y, Lin B, Luo F, Zhang C, Chen S, Li Y, Xu A (2010) A unique feature of Toll/IL-1 receptor domain-containing adaptor protein is partially responsible for lipopolysaccharide insensitivity in zebrafish with a highly conserved function of MyD88. *J Immunol* 185:3391-3400.
- Lopez-Diez R, Rastrojo A, Villate O, Aguado B (2013) Complex tissue-specific patterns and distribution of multiple RAGE splice variants in different mammals. *Genome biology and evolution* 5:2420-2435.
- Lotfi R, Herzog GI, DeMarco RA, Beer-Stolz D, Lee JJ, Rubartelli A, Schrezenmeier H, Lotze MT (2009) Eosinophils oxidize damage-associated molecular pattern molecules derived from stressed cells. *J Immunol* 183:5023-5031.
- Luan ZG, Zhang H, Yang PT, Ma XC, Zhang C, Guo RX (2010) HMGB1 activates nuclear factor-kappaB signaling by RAGE and increases the production of TNF- α in human umbilical vein endothelial cells. *Immunobiology* 215:956-962.

- Lucas K, Maes M (2013) Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. *Molecular neurobiology* 48:190-204.
- Lucas SM, Rothwell NJ, Gibson RM (2006) The role of inflammation in CNS injury and disease. *British journal of pharmacology* 147 Suppl 1:S232-240.
- Luo L, Jin Y, Kim ID, Lee JK (2014) Glycyrrhizin suppresses HMGB1 inductions in the hippocampus and subsequent accumulation in serum of a kainic acid-induced seizure mouse model. *Cellular and molecular neurobiology* 34:987-997.
- Ma C, LaMotte RH (2005) Enhanced excitability of dissociated primary sensory neurons after chronic compression of the dorsal root ganglion in the rat. *Pain* 113:106-112.
- Madera-Salcedo IK, Cruz SL, Gonzalez-Espinosa C (2013) Morphine prevents lipopolysaccharide-induced TNF secretion in mast cells blocking IkappaB kinase activation and SNAP-23 phosphorylation: correlation with the formation of a beta-arrestin/TRAF6 complex. *J Immunol* 191:3400-3409.
- Magna M, Pisetsky DS (2014) The role of HMGB1 in the pathogenesis of inflammatory and autoimmune diseases. *Mol Med* 20:138-146.
- Mangalmurti NS, Friedman JL, Wang LC, Stolz DB, Muthukumaran G, Siegel DL, Schmidt AM, Lee JS, Albelda SM (2012) The Receptor for Advanced Glycation End Products Mediates Lung Endothelial Activation by RBCs. *American journal of physiology Lung cellular and molecular physiology*.

- Mansell A, Brint E, Gould JA, O'Neill LA, Hertzog PJ (2004) Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF-kappaB activation by toll-like receptor (TLR)-2 and TLR4. *J Biol Chem* 279:37227-37230.
- Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. *Nature* 454:436-444.
- Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. *Nature medicine* 16:413-419.
- McFarland HF, Martin R (2007) Multiple sclerosis: a complicated picture of autoimmunity. *Nature immunology* 8:913-919.
- McGettrick AF, O'Neill LA (2010) Localisation and trafficking of Toll-like receptors: an important mode of regulation. *Current opinion in immunology* 22:20-27.
- Medzhitov R (2010) Inflammation 2010: new adventures of an old flame. *Cell* 140:771-776.
- Medzhitov R, Janeway CA, Jr. (1997) Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295-298.
- Medzhitov R, Janeway CA, Jr. (1998) Innate immune recognition and control of adaptive immune responses. *Seminars in immunology* 10:351-353.
- Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394-397.

- Merenmies J, Pihlaskari R, Laitinen J, Wartiovaara J, Rauvala H (1991) 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. *The Journal of biological chemistry* 266:16722-16729.
- Meseguer V, Alpizar YA, Luis E, Tajada S, Denlinger B, Fajardo O, Manenschijn JA, Fernandez-Pena C, Talavera A, Kichko T, Navia B, Sanchez A, Senaris R, Reeh P, Perez-Garcia MT, Lopez-Lopez JR, Voets T, Belmonte C, Talavera K, Viana F (2014) TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. *Nat Commun* 5:3125.
- Miller MC, Tavares R, Johanson CE, Hovanesian V, Donahue JE, Gonzalez L, Silverberg GD, Stopa EG (2008) Hippocampal RAGE immunoreactivity in early and advanced Alzheimer's disease. *Brain research* 1230:273-280.
- Mina-Osorio P, Rosas-Ballina M, Valdes-Ferrer SI, Al-Abed Y, Tracey KJ, Diamond B (2012) Neural signaling in the spleen controls B-cell responses to blood-borne antigen. *Mol Med* 18:618-627.
- Mitkova E, Ugrinova I, Pashev IG, Pasheva EA (2005) The inhibitory effect of HMGB-1 protein on the repair of cisplatin-damaged DNA is accomplished through the acidic domain. *Biochemistry* 44:5893-5898.
- Mohammad G, Siddiquei MM, Othman A, Al-Shabrawey M, Abu El-Asrar AM (2012) High-mobility group box-1 protein activates inflammatory signaling pathway components and disrupts retinal vascular-barrier in the diabetic retina. *Experimental eye research*.

- Mollica L, De Marchis F, Spitaleri A, Dallacosta C, Pennacchini D, Zamai M, Agresti A, Trisciuglio L, Musco G, Bianchi ME (2007) Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. *Chemistry & biology* 14:431-441.
- Morris DB (1998) An invisible history of pain: early 19th-century Britain and America. *The Clinical journal of pain* 14:191-196.
- Muhammad S, Barakat W, Stoyanov S, Murikinati S, Yang H, Tracey KJ, Bendszus M, Rossetti G, Nawroth PP, Bierhaus A, Schwaninger M (2008) The HMGB1 receptor RAGE mediates ischemic brain damage. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28:12023-12031.
- Muzio M, Natoli G, Sacconi S, Levrero M, Mantovani A (1998) The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *The Journal of experimental medicine* 187:2097-2101.
- Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M, Miyake K (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature immunology* 3:667-672.
- Nagatani G, Nomoto M, Takano H, Ise T, Kato K, Imamura T, Izumi H, Makishima K, Kohno K (2001) Transcriptional activation of the human HMG1 gene in cisplatin-resistant human cancer cells. *Cancer research* 61:1592-1597.
- Nagpal K, Plantinga TS, Wong J, Monks BG, Gay NJ, Netea MG, Fitzgerald KA, Golenbock DT (2009) A TIR domain variant of MyD88 adapter-like (Mal)/TIRAP results in loss

of MyD88 binding and reduced TLR2/TLR4 signaling. J Biol Chem 284:25742-25748.

Nakamura Y, Morioka N, Abe H, Zhang FF, Hisaoka-Nakashima K, Liu K, Nishibori M, Nakata Y (2013) Neuropathic pain in rats with a partial sciatic nerve ligation is alleviated by intravenous injection of monoclonal antibody to high mobility group box-1. PLoS One 8:e73640.

Nathan C, Ding A (2010) Nonresolving inflammation. Cell 140:871-882.

Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, Elliston K, Stern D, Shaw A (1992) Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. The Journal of biological chemistry 267:14998-15004.

Nejentsev S, Thye T, Szeszko JS, Stevens H, Balabanova Y, Chinbuah AM, Hibberd M, van de Vosse E, Alisjahbana B, van Crevel R, Ottenhoff TH, Png E, Drobniewski F, Todd JA, Seielstad M, Horstmann RD (2008) Analysis of association of the TIRAP (MAL) S180L variant and tuberculosis in three populations. Nature genetics 40:261-262; author reply 262-263.

Nijland R, Hofland T, van Strijp JA (2014) Recognition of LPS by TLR4: potential for anti-inflammatory therapies. Marine drugs 12:4260-4273.

O'Connor KA, Hansen MK, Rachal Pugh C, Deak MM, Biedenkapp JC, Milligan ED, Johnson JD, Wang H, Maier SF, Tracey KJ, Watkins LR (2003) Further characterization of high mobility group box 1 (HMGB1) as a proinflammatory cytokine: central nervous system effects. Cytokine 24:254-265.

- O'Neill J, Brock C, Olesen AE, Andresen T, Nilsson M, Dickenson AH (2012) Unravelling the mystery of capsaicin: a tool to understand and treat pain. *Pharmacological reviews* 64:939-971.
- O'Neill LA, Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature reviews Immunology* 7:353-364.
- O'Neill LA, Fitzgerald KA, Bowie AG (2003) The Toll-IL-1 receptor adaptor family grows to five members. *Trends in immunology* 24:286-290.
- Ochoa-Cortes F, Ramos-Lomas T, Miranda-Morales M, Spreadbury I, Ibeakanma C, Barajas-Lopez C, Vanner S (2010) Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. *American journal of physiology Gastrointestinal and liver physiology* 299:G723-732.
- Oeppen J, Vaupel JW (2002) Demography. Broken limits to life expectancy. *Science* 296:1029-1031.
- Ohto U, Fukase K, Miyake K, Satow Y (2007) Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. *Science* 316:1632-1634.
- Okuma Y, Liu K, Wake H, Liu R, Nishimura Y, Hui Z, Teshigawara K, Haruma J, Yamamoto Y, Yamamoto H, Date I, Takahashi HK, Mori S, Nishibori M (2014) Glycyrrhizin inhibits traumatic brain injury by reducing HMGB1-RAGE interaction. *Neuropharmacology* 85:18-26.
- Okun E, Griffioen KJ, Mattson MP (2011) Toll-like receptor signaling in neural plasticity and disease. *Trends in neurosciences* 34:269-281.

- Ostberg T, Wahamaa H, Palmblad K, Ito N, Stridh P, Shoshan M, Lotze MT, Harris HE, Andersson U (2008) Oxaliplatin retains HMGB1 intranuclearly and ameliorates collagen type II-induced arthritis. *Arthritis research & therapy* 10:R1.
- Otoshi K, Kikuchi S, Kato K, Sekiguchi M, Konno S (2011) Anti-HMGB1 neutralization antibody improves pain-related behavior induced by application of autologous nucleus pulposus onto nerve roots in rats. *Spine (Phila Pa 1976)* 36:E692-698.
- Ott C, Jacobs K, Haucke E, Navarrete Santos A, Grune T, Simm A (2014) Role of advanced glycation end products in cellular signaling. *Redox biology* 2:411-429.
- Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S (2012) Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nature medicine* 18:1279-1285.
- Palone F, Vitali R, Cucchiara S, Pierdomenico M, Negroni A, Aloï M, Nuti F, Felice C, Armuzzi A, Stronati L (2014) Role of HMGB1 as a suitable biomarker of subclinical intestinal inflammation and mucosal healing in patients with inflammatory bowel disease. *Inflammatory bowel diseases* 20:1448-1457.
- Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458:1191-1195.
- Park HJ, Stokes JA, Corr M, Yaksh TL (2013) Toll-like receptor signaling regulates cisplatin-induced mechanical allodynia in mice. *Cancer Chemother Pharmacol*.
- Parkkinen J, Raulo E, Merenmies J, Nolo R, Kajander EO, Baumann M, Rauvala H (1993) Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced

- expression in transformed cells, leading edge localization, and interactions with plasminogen activation. *The Journal of biological chemistry* 268:19726-19738.
- Paterson HM, Murphy TJ, Purcell EJ, Shelley O, Kriynovich SJ, Lien E, Mannick JA, Lederer JA (2003) Injury primes the innate immune system for enhanced Toll-like receptor reactivity. *J Immunol* 171:1473-1483.
- Pitkanen A, Lukasiuk K (2009) Molecular and cellular basis of epileptogenesis in symptomatic epilepsy. *Epilepsy & behavior : E&B* 14 Suppl 1:16-25.
- Polanska E, Pospisilova S, Stros M (2014) Binding of histone H1 to DNA is differentially modulated by redox state of HMGB1. *PloS one* 9:e89070.
- Qiu J, Xu J, Zheng Y, Wei Y, Zhu X, Lo EH, Moskowitz MA, Sims JR (2010) High-mobility group box 1 promotes metalloproteinase-9 upregulation through Toll-like receptor 4 after cerebral ischemia. *Stroke* 41:2077-2082.
- Rallabhandi P, Nhu QM, Toshchakov VY, Piao W, Medvedev AE, Hollenberg MD, Fasano A, Vogel SN (2008) Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. *The Journal of biological chemistry* 283:24314-24325.
- Rathinam VA, Fitzgerald KA (2013) Immunology: Lipopolysaccharide sensing on the inside. *Nature* 501:173-175.
- Rauvala H, Pihlaskari R (1987) Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. *The Journal of biological chemistry* 262:16625-16635.

- Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A (2005) The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America* 102:9577-9582.
- Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF (1998) A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci U S A* 95:588-593.
- Rohde D, Ritterhoff J, Voelkers M, Katus HA, Parker TG, Most P (2010) S100A1: a multifaceted therapeutic target in cardiovascular disease. *Journal of cardiovascular translational research* 3:525-537.
- Rolan P, Hutchinson M, Johnson K (2009) Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. *Expert opinion on pharmacotherapy* 10:2897-2904.
- Rong LL, Trojaborg W, Qu W, Kostov K, Yan SD, Gooch C, Szabolcs M, Hays AP, Schmidt AM (2004a) Antagonism of RAGE suppresses peripheral nerve regeneration. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18:1812-1817.
- Rong LL, Yan SF, Wendt T, Hans D, Pachydaki S, Bucciarelli LG, Adebayo A, Qu W, Lu Y, Kostov K, Lalla E, Yan SD, Gooch C, Szabolcs M, Trojaborg W, Hays AP, Schmidt AM (2004b) RAGE modulates peripheral nerve regeneration via recruitment of both inflammatory and axonal outgrowth pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18:1818-1825.

- Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, Tusche MW, Pavlov VA, Andersson U, Chavan S, Mak TW, Tracey KJ (2011) Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* 334:98-101.
- Rossi AG, Hallett JM, Sawatzky DA, Teixeira MM, Haslett C (2007) Modulation of granulocyte apoptosis can influence the resolution of inflammation. *Biochemical Society transactions* 35:288-291.
- Rouhiainen A, Kuja-Panula J, Tumova S, Rauvala H (2013) RAGE-Mediated Cell Signaling. *Methods Mol Biol* 963:239-263.
- Rouhiainen A, Kuja-Panula J, Wilkman E, Pakkanen J, Stenfors J, Tuominen RK, Lepantalo M, Carpen O, Parkkinen J, Rauvala H (2004) Regulation of monocyte migration by amphoterin (HMGB1). *Blood* 104:1174-1182.
- Roy S, Cain KJ, Chapin RB, Charboneau RG, Barke RA (1998) Morphine modulates NF kappa B activation in macrophages. *Biochem Biophys Res Commun* 245:392-396.
- Rubartelli A, Lotze MT (2007) Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends in immunology* 28:429-436.
- Sahu D, Debnath P, Takayama Y, Iwahara J (2008) Redox properties of the A-domain of the HMGB1 protein. *FEBS Lett* 582:3973-3978.
- Saitoh S, Akashi S, Yamada T, Tanimura N, Matsumoto F, Fukase K, Kusumoto S, Kosugi A, Miyake K (2004) Ligand-dependent Toll-like receptor 4 (TLR4)-oligomerization is directly linked with TLR4-signaling. *Journal of endotoxin research* 10:257-260.

- Sakaguchi M, Murata H, Yamamoto K, Ono T, Sakaguchi Y, Motoyama A, Hibino T, Kataoka K, Huh NH (2011) TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding. *PloS one* 6:e23132.
- Saleh A, Smith DR, Tessler L, Mateo AR, Martens C, Schartner E, Van der Ploeg R, Toth C, Zochodne DW, Fernyhough P (2013) Receptor for advanced glycation end-products (RAGE) activates divergent signaling pathways to augment neurite outgrowth of adult sensory neurons. *Experimental neurology* 249:149-159.
- Sankowski R, Mader S, Valdes-Ferrer SI (2015) Systemic inflammation and the brain: novel roles of genetic, molecular, and environmental cues as drivers of neurodegeneration. *Frontiers in cellular neuroscience* 9:28.
- Santos-Sierra S, Deshmukh SD, Kalnitski J, Kuenzi P, Wymann MP, Golenbock DT, Henneke P (2009) Mal connects TLR2 to PI3Kinase activation and phagocyte polarization. *The EMBO journal* 28:2018-2027.
- Savill J (1997) Apoptosis in resolution of inflammation. *Journal of leukocyte biology* 61:375-380.
- Schaible HG (2014) Nociceptive neurons detect cytokines in arthritis. *Arthritis research & therapy* 16:470.
- Schaper J, Wagner A, Enigk F, Brell B, Mousa SA, Habazettl H, Schafer M (2013) Regional sympathetic blockade attenuates activation of intestinal macrophages and reduces gut barrier failure. *Anesthesiology* 118:134-142.
- Schiraldi M, Raucci A, Munoz LM, Livoti E, Celona B, Venereau E, Apuzzo T, De Marchis F, Pedotti M, Bachi A, Thelen M, Varani L, Mellado M, Proudfoot A, Bianchi ME,

- Uguccioni M (2012) HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *The Journal of experimental medicine* 209:551-563.
- Schmidt AM, Yan SD, Yan SF, Stern DM (2001) The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *The Journal of clinical investigation* 108:949-955.
- Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285:1569-1572.
- Serhan CN (2008) Controlling the resolution of acute inflammation: a new genus of dual anti-inflammatory and proresolving mediators. *Journal of periodontology* 79:1520-1526.
- Sessa L, Gatti E, Zeni F, Antonelli A, Catucci A, Koch M, Pompilio G, Fritz G, Raucci A, Bianchi ME (2014) The receptor for advanced glycation end-products (RAGE) is only present in mammals, and belongs to a family of cell adhesion molecules (CAMs). *PloS one* 9:e86903.
- Sha Y, Zmijewski J, Xu Z, Abraham E (2008) HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *J Immunol* 180:2531-2537.
- Shibasaki M, Sasaki M, Miura M, Mizukoshi K, Ueno H, Hashimoto S, Tanaka Y, Amaya F (2010) Induction of high mobility group box-1 in dorsal root ganglion contributes to pain hypersensitivity after peripheral nerve injury. *Pain* 149:514-521.

- Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine* 189:1777-1782.
- Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ (2010) HMGB1 and RAGE in inflammation and cancer. *Annual review of immunology* 28:367-388.
- Skolnick P, Davis H, Arnette D, Deaver D (2014) Translational potential of naloxone and naltrexone as TLR4 antagonists. *Trends in pharmacological sciences* 35:431-432.
- Smith MT (2000) Neuroexcitatory effects of morphine and hydromorphone: evidence implicating the 3-glucuronide metabolites. *Clinical and experimental pharmacology & physiology* 27:524-528.
- Sorci G, Riuzzi F, Giambanco I, Donato R (2012) RAGE in tissue homeostasis, repair and regeneration. *Biochimica et biophysica acta*.
- Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin JS, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS (2011) Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31:15450-15454.
- Sousa MM, Du Yan S, Fernandes R, Guimaraes A, Stern D, Saraiva MJ (2001) Familial amyloid polyneuropathy: receptor for advanced glycation end products-dependent triggering of neuronal inflammatory and apoptotic pathways. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21:7576-7586.

- Spahn V, Stein C, Zollner C (2014) Modulation of transient receptor vanilloid 1 activity by transient receptor potential ankyrin 1. *Mol Pharmacol* 85:335-344.
- Stavropoulou E, Zis P, Vadalouca A (2014) The use of intravenous lidocaine in trigeminal neuralgia pain relief and palliative care unit. *Pain physician* 17:E235-236.
- Stemmer C, Leeming DJ, Franssen L, Grimm R, Grasser KD (2003) Phosphorylation of maize and Arabidopsis HMGB proteins by protein kinase CK2alpha. *Biochemistry* 42:3503-3508.
- Stemmer C, Schwander A, Bauw G, Fojan P, Grasser KD (2002) Protein kinase CK2 differentially phosphorylates maize chromosomal high mobility group B (HMGB) proteins modulating their stability and DNA interactions. *The Journal of biological chemistry* 277:1092-1098.
- Sterenczak KA, Nolte I, Murua Escobar H (2013) RAGE splicing variants in mammals. *Methods Mol Biol* 963:265-276.
- Sterner R, Vidali G, Allfrey VG (1979) Studies of acetylation and deacetylation in high mobility group proteins. Identification of the sites of acetylation in HMG-1. *The Journal of biological chemistry* 254:11577-11583.
- Stevens CW, Aravind S, Das S, Davis RL (2013) Pharmacological characterization of LPS and opioid interactions at the toll-like receptor 4. *British journal of pharmacology* 168:1421-1429.
- Stokes JA, Corr M, Yaksh TL (2011) Transient tactile allodynia following intrathecal puncture in mouse: contributions of Toll-like receptor signaling. *Neurosci Lett* 504:215-218.

- Stokes JA, Corr M, Yaksh TL (2013) Spinal toll-like receptor signaling and nociceptive processing: regulatory balance between TIRAP and TRIF cascades mediated by TNF and IFNbeta. *Pain* 154:733-742.
- Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 112:819-829.
- Strakhova MI, Desiree-Brderson J (2013) Antibodies to receptor of advanced glycation end products (RAGE) and uses thereof. (Office, U. S. P. a. T., ed) United States: Abbvie Inc., North Chicago, IL
- Stros M (2010) HMGB proteins: interactions with DNA and chromatin. *Biochimica et biophysica acta* 1799:101-113.
- Sun Q, Wu W, Hu YC, Li H, Zhang D, Li S, Li W, Li WD, Ma B, Zhu JH, Zhou ML, Hang CH (2014) Early release of high-mobility group box 1 (HMGB1) from neurons in experimental subarachnoid hemorrhage in vivo and in vitro. *Journal of neuroinflammation* 11:106.
- Sunden-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, Lee ML, Andersson J, Tokics L, Treutiger CJ (2005) Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. *Critical care medicine* 33:564-573.

- Takeda K, Akira S (2005) Toll-like receptors in innate immunity. *International immunology* 17:1-14.
- Tang D, Billiar TR, Lotze MT (2012a) A Janus tale of two active high mobility group box 1 (HMGB1) redox states. *Mol Med* 18:1360-1362.
- Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT (2012b) PAMPs and DAMPs: signals that spur autophagy and immunity. *Immunological reviews* 249:158-175.
- Tang D, Kang R, Zeh HJ, 3rd, Lotze MT (2010) High-mobility group box 1 and cancer. *Biochimica et biophysica acta* 1799:131-140.
- Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proceedings of the National Academy of Sciences of the United States of America* 102:5856-5861.
- Tesana S, Thapsripair P, Thammasiri C, Prasopdee S, Suwannatrai A, Haraui S, Piratae S, Khampoosa P, Kulsantiwong J, Donthaisong C, Chalokepanrat P, Viyanant V, Malone JB (2012) Effects of Bayluscide on *Bithynia siamensis goniomphalos*, the first intermediate host of the human liver fluke, *Opisthorchis viverrini*, in laboratory and field trials. *Parasitology international* 61:52-55.
- Thomas JO (2001) HMG1 and 2: architectural DNA-binding proteins. *Biochemical Society transactions* 29:395-401.
- Thomas JO, Travers AA (2001) HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends in biochemical sciences* 26:167-174.

- Topalova D, Ugrinova I, Pashev IG, Pasheva EA (2008) HMGB1 protein inhibits DNA replication in vitro: a role of the acetylation and the acidic tail. The international journal of biochemistry & cell biology 40:1536-1542.
- Toshchakov VY, Fenton MJ, Vogel SN (2007) Cutting Edge: Differential inhibition of TLR signaling pathways by cell-permeable peptides representing BB loops of TLRs. J Immunol 178:2655-2660.
- Toshchakov VY, Szmazinski H, Couture LA, Lakowicz JR, Vogel SN (2011) Targeting TLR4 signaling by TLR4 Toll/IL-1 receptor domain-derived decoy peptides: identification of the TLR4 Toll/IL-1 receptor domain dimerization interface. J Immunol 186:4819-4827.
- Tse KH, Chow KB, Leung WK, Wong YH, Wise H (2014) Lipopolysaccharide differentially modulates expression of cytokines and cyclooxygenases in dorsal root ganglion cells via Toll-like receptor-4 dependent pathways. Neuroscience 267:241-251.
- Tsung A, Tohme S, Billiar TR (2014) High-mobility group box-1 in sterile inflammation. Journal of internal medicine 276:425-443.
- Ulloa L, Tracey KJ (2005) The "cytokine profile": a code for sepsis. Trends Mol Med 11:56-63.
- Van-Assche T, Huygelen V, Crabtree MJ, Antoniades C (2011) Gene therapy targeting inflammation in atherosclerosis. Current pharmaceutical design 17:4210-4223.
- van Zoelen MA, Achouiti A, van der Poll T (2011) RAGE during infectious diseases. Frontiers in bioscience 3:1119-1132.

- Vaupel JW, KG VK (2005) [The remarkable rise in life expectancy and how it will affect medicine]. Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz 48:586-592.
- Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, Liu J, Antonelli A, Preti A, Raeli L, Shams SS, Yang H, Varani L, Andersson U, Tracey KJ, Bachi A, Uguccioni M, Bianchi ME (2012a) Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. The Journal of experimental medicine 209:1519-1528.
- Venereau E, Schiraldi M, Uguccioni M, Bianchi ME (2012b) HMGB1 and leukocyte migration during trauma and sterile inflammation. Mol Immunol.
- Vezzoli M, Castellani P, Campana L, Corna G, Bosurgi L, Manfredi AA, Bianchi ME, Rubartelli A, Rovere-Querini P (2010) Redox remodeling: a candidate regulator of HMGB1 function in injured skeletal muscle. Ann N Y Acad Sci 1209:83-90.
- Vezzoli M, Castellani P, Corna G, Castiglioni A, Bosurgi L, Monno A, Brunelli S, Manfredi AA, Rubartelli A, Rovere-Querini P (2011) High-mobility group box 1 release and redox regulation accompany regeneration and remodeling of skeletal muscle. Antioxid Redox Signal 15:2161-2174.
- Vilceanu D, Stucky CL (2010) TRPA1 mediates mechanical currents in the plasma membrane of mouse sensory neurons. PLoS One 5:e12177.
- Vitali R, Stronati L, Negroni A, Di Nardo G, Pierdomenico M, del Giudice E, Rossi P, Cucchiara S (2011) Fecal HMGB1 is a novel marker of intestinal mucosal

- inflammation in pediatric inflammatory bowel disease. *The American journal of gastroenterology* 106:2029-2040.
- Voscopoulos C, Lema M (2010) When does acute pain become chronic? *British journal of anaesthesia* 105 Suppl 1:i69-85.
- Wadachi R, Hargreaves KM (2006) Trigeminal nociceptors express TLR-4 and CD14: a mechanism for pain due to infection. *J Dent Res* 85:49-53.
- Waldburger JM, Firestein GS (2009) Garden of therapeutic delights: new targets in rheumatic diseases. *Arthritis research & therapy* 11:206.
- Waller BJ, Alberts AS (2003) The formins: active scaffolds that remodel the cytoskeleton. *Trends in cell biology* 13:435-446.
- Wang J, Tochio N, Takeuchi A, Uewaki J, Kobayashi N, Tate S (2013) Redox-sensitive structural change in the A-domain of HMGB1 and its implication for the binding to cisplatin modified DNA. *Biochemical and biophysical research communications* 441:701-706.
- Wang X, Loram LC, Ramos K, de Jesus AJ, Thomas J, Cheng K, Reddy A, Somogyi AA, Hutchinson MR, Watkins LR, Yin H (2012) Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc Natl Acad Sci U S A* 109:6325-6330.
- Watanabe N, Madaule P, Reid T, Ishizaki T, Watanabe G, Kakizuka A, Saito Y, Nakao K, Jockusch BM, Narumiya S (1997) p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *The EMBO journal* 16:3044-3056.

- Weber DJ, Gracon AS, Ripsch MS, Fisher AJ, Cheon BM, Pandya PH, Vittal R, Capitano ML, Kim Y, Allette YM, Riley AA, McCarthy BP, Territo PR, Hutchins GD, Broxmeyer HE, Sandusky GE, White FA, Wilkes DS (2014) The HMGB1-RAGE axis mediates traumatic brain injury-induced pulmonary dysfunction in lung transplantation. *Science translational medicine* 6:252ra124.
- White FA, Bhangoo SK, Miller RJ (2005) Chemokines: integrators of pain and inflammation. *Nature reviews Drug discovery* 4:834-844.
- White FA, Jung H, Miller RJ (2007) Chemokines and the pathophysiology of neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America* 104:20151-20158.
- White FA, Wilson NM (2008) Chemokines as pain mediators and modulators. *Current opinion in anaesthesiology* 21:580-585.
- Wilson NM, Jung H, Ripsch MS, Miller RJ, White FA (2011) CXCR4 signaling mediates morphine-induced tactile hyperalgesia. *Brain, behavior, and immunity* 25:565-573.
- Woller SA, Corr M, Yaksh TL (2015) Differences in cisplatin-induced mechanical allodynia in male and female mice. *Eur J Pain*.
- Woo JR, Kim, S., Shoelson, S.E., Park, S. (2012) X-ray Crystallographic Structure of TIR-Domain from the Human TIR-Domain Containing Adaptor Protein/MyD88-Adaptor-Like Protein (TIRAP/MAL). *Bulletin - Korean Chemical Society* 33.
- Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. *Science* 288:1765-1769.

- Xie J, Mendez JD, Mendez-Valenzuela V, Aguilar-Hernandez MM (2013) Cellular signaling of the receptor for advanced glycation end products (RAGE). Cellular signalling.
- Xue J, Rai V, Singer D, Chabierski S, Xie J, Reverdatto S, Burz DS, Schmidt AM, Hoffmann R, Shekhtman A (2011) Advanced glycation end product recognition by the receptor for AGEs. Structure 19:722-732.
- Yaksh TL, Noueihed RY, Durant PA (1986) Studies of the pharmacology and pathology of intrathecally administered 4-anilinopiperidine analogues and morphine in the rat and cat. Anesthesiology 64:54-66.
- Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nature immunology 4:1144-1150.
- Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol 169:6668-6672.
- Yamamoto M, Takeda K, Akira S (2004) TIR domain-containing adaptors define the specificity of TLR signaling. Molecular immunology 40:861-868.
- Yamamoto Y, Harashima A, Saito H, Tsuneyama K, Munesue S, Motoyoshi S, Han D, Watanabe T, Asano M, Takasawa S, Okamoto H, Shimura S, Karasawa T, Yonekura H, Yamamoto H (2011) Septic shock is associated with receptor for advanced glycation end products ligation of LPS. J Immunol 186:3248-3257.

- Yan SD, Bierhaus A, Nawroth PP, Stern DM (2009) RAGE and Alzheimer's disease: a progression factor for amyloid-beta-induced cellular perturbation? *Journal of Alzheimer's disease* : JAD 16:833-843.
- Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM (1996) RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature* 382:685-691.
- Yang H, Antoine DJ, Andersson U, Tracey KJ (2013) The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. *Journal of leukocyte biology* 93:865-873.
- Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas-Ballina M, Al-Abed Y, Akira S, Bierhaus A, Erlandsson-Harris H, Andersson U, Tracey KJ (2010) A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proceedings of the National Academy of Sciences of the United States of America* 107:11942-11947.
- Yang H, Lundback P, Ottosson L, Erlandsson-Harris H, Venereau E, Bianchi ME, Al-Abed Y, Andersson U, Tracey KJ, Antoine DJ (2012) Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). *Mol Med* 18:250-259.
- Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Wang H, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ (2004) Reversing established sepsis with antagonists of endogenous

high-mobility group box 1. Proceedings of the National Academy of Sciences of the United States of America 101:296-301.

Yang QW, Lu FL, Zhou Y, Wang L, Zhong Q, Lin S, Xiang J, Li JC, Fang CQ, Wang JZ (2011) HMBG1 mediates ischemia-reperfusion injury by TRIF-adaptor independent Toll-like receptor 4 signaling. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 31:593-605.

Youn JH, Shin JS (2006) Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. J Immunol 177:7889-7897.

Zandarashvili L, Sahu D, Lee K, Lee YS, Singh P, Rajarathnam K, Iwahara J (2013) Real-time kinetics of high-mobility group box 1 (HMGB1) oxidation in extracellular fluids studied by in situ protein NMR spectroscopy. J Biol Chem 288:11621-11627.

Zeng L, Zhang AQ, Gu W, Zhou J, Zhang LY, Du DY, Zhang M, Wang HY, Yan J, Yang C, Jiang JX (2012) Identification of haplotype tag single nucleotide polymorphisms within the receptor for advanced glycation end products gene and their clinical relevance in patients with major trauma. Crit Care 16:R131.

Zhang J, Takahashi HK, Liu K, Wake H, Liu R, Maruo T, Date I, Yoshino T, Ohtsuka A, Mori S, Nishibori M (2011) Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats. Stroke 42:1420-1428.

Zhang X, Huang J, McNaughton PA (2005) NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. The EMBO journal 24:4211-4223.

Zou JY, Crews FT (2014) Release of neuronal HMGB1 by ethanol through decreased HDAC activity activates brain neuroimmune signaling. PloS one 9:e87915.

Zurolo E, Iyer A, Maroso M, Carbonell C, Anink JJ, Ravizza T, Fluiter K, Spliet WG, van Rijen PC, Vezzani A, Aronica E (2011) Activation of Toll-like receptor, RAGE and HMGB1 signalling in malformations of cortical development. Brain : a journal of neurology 134:1015-1032.

Curriculum Vitae

EDUCATION

Indiana University

Ph.D. in Anatomy and Cell Biology

2012 – 2015

University of Maryland Baltimore County

B.S. in Biological Sciences

2006 – 2010

AWARDS

CTSI Pre-doctoral Training Award in Translational Research

2014

Travel Award Recipient for 10th Annual APSA Meeting

2014

Outstanding Poster Presentation at ABR Conference

2008

John H. Sr. and Althea Griner Scholar (UMBC)

2007 – 2008

Baltimore's Most Amazing Youth, Urban Leadership Institute

2006

RESEARCH EXPERIENCE

Indiana University School of Medicine, Graduate Studies – Dr. Fletcher White

The Role of HMGB1 in the Neuropathic Pain

2012 – 2015

The project focuses on the investigation of the mechanism behind the neuronal signaling cascades responsible for the development of chronic pain and its related sequelae. Specifically, this revolves around the function of the RAGE receptor, its downstream affects, and its relationship with other neuroinflammatory cascades, such as those associated with the TLR4 receptor.

University of Maryland Baltimore County, Undergraduate Studies – Dr. Rachel Brewster

Investigation of Migrating Neural Cells in Zebrafish

2008 – 2010

The goal of the project was to determine whether the transmembrane protein Neogenin mediates polarized cell migration during neurulation, in order to increase understanding of the molecular mechanisms that regulate neurulation, and provide putative candidate genes implicated in the etiology of human neural tube birth defects.

University of Miami: Miller School of Medicine, Undergraduate Studies – Dr. Kent Lai

Identification of Novel Antimicrobials

2008

The project centered on finding new defenses against microbial pathogens has become increasingly important due to the growing issue of new antibiotic resistant

strains. This was done via the manipulation of the non-mevalonate pathway of isoprenoid biosynthesis using gene knockdown and small molecule inhibitors.

Johns Hopkins University / National Institutes of Health – Dr. Joan Bailey-Wilson

GWAS Studies of Cleft/Lip Palate

2007

The research focused around computational analysis of genetic samples obtained from various pedigrees expressing cleft lip and palate in order to identify new targets for bench study, and to increase the translation rate of the data from bench to bedside.

PUBLICATIONS AND PAPERS

The HMGB1-RAGE Inflammatory Pathway: Implications for Brain Injury Induced Pulmonary Dysfunction.

Weber DJ, Allette YM, Wilkes DS, White FA. *Antioxidants and Redox Signaling*. 2015 (In press)

Identification of a functional interaction of HMGB1 with Receptor for Advanced Glycation End-Products in a model of neuropathic pain.

Allette YM, Due MR, Wilson SM, Feldman P, Ripsch MS, Khanna R, White FA. *Brain Behav Immun*. 2014 Nov;42:169-77.

Carbamazepine potentiates the effectiveness of morphine in a rodent model of neuropathic pain.

Due MR, Xang XF, Allette YM, Randolph AL, Ripsch MS, Wilson SM, Dustrude ET, Khanna R, White FA. *PLoS One*. 2014 Sep 15;9(9):e107399.

The HMGB1-RAGE axis mediates traumatic brain injury-induced pulmonary dysfunction in lung transplantation.

Weber DJ, Gracon AS, Ripsch MS, Fisher AJ, Cheon BM, Pandya PH, Wittal R, Capitano ML, Kim Y, Allette YM, Riley AA, McCarthy BP, Territo PR, Hutchins GD, Broxmeyer HE, Sandusky GE, White FA, Wilkes DS. *Sci Transl Med*. 2014 Sep 3;6(252):252ra124.

Acrolein involvement in sensory and behavioral hypersensitivity following spinal cord injury in the rat.

Due MR, Park J, Zheng L, Walls M, Allette YM, White FA, Shi R. *J Neurochem*. 2013 Oct 21.

The Role of the Neuronal Toll-like Receptor 4 in Neuropathic Pain and Opioid-induced Hyperalgesia

Due MR, Allette YM, White FA. (2013) In: Toth C, Moulin D (eds.), *Neuropathic Pain: Causes, Management and Understanding*.

Suppression of pain-related behavior in two distinct rodent models of peripheral neuropathy by a homopolyarginine-conjugated CRMP2 peptide.

Ju W, Li Q, Allette YM, Ripsch MS, White FA, Khanna R.

J Neurochem. 2012 Oct 26.

Identification of novel small molecule inhibitors of 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) kinase of Gram-negative bacteria.

Tang M, Odejinmi SI, Allette YM, Vankayalapati H, Lai K.

Bioorg Med Chem. 2011 Oct 1;19(19):5886-95.

ACADEMIC CONFERENCES & MEETINGS

- 2014 Gill Neuroscience Symposium, Bloomington, IN – September 2014
 - Poster Presentation
- Indiana CTSI 2014 Annual Meeting, Indianapolis IN – September 2014
 - Poster Presentation
- 10th APSA (American Physician Scientists Association) Meeting, Chicago, IL – April 2014
 - Travel Grant Recipient
 - Poster Presentation
- 4th International Congress on Neuropathic Pain (NeuPSIG), Toronto, Ontario – May 2013
 - Poster Presentation
- Ronald E. McNair Texas National Conference, Denton, TX – February 2009
 - Oral Presentation
- Annual Biomedical Research Conference for Minority Students (ABRCMS), Orlando, FL – November 2008
 - Poster Presentation
- The Leadership Alliance Summer Conference, Hartford, CT – August 2008
 - Poster Presentation

MEMBERSHIPS

American Physician Scientists Association (APSA) Member
International Association for the Study of Pain (IASP) Member
Golden Key Honour Society Graduate Member
MARC U*Star Scholar